CHARACTERISING CHEMICAL CO-EXPOSURES IN EU TO SUPPORT A COMBINED EXPOSURE ASSESSMENT STRATEGY

TECHNICAL ANNEXES A-D

27/10/2021

Environment

Karel Viaene

Frederik Verdonck

Linh-Dan Ngo

Charlotte Nys

Marnix Vangheluwe

ARCHE Consulting
Liefkensstraat 35D
B-9032 Wondelgem, Belgium

Human health

Katleen De Brouwere

Jos Bessems

Eva Govarts

Jirka Cops

Flemish Institute for Technological Research ("VITO")
Boeretang 200
BE-2400 Mol, Belgium

Contact

karel.viaene@arche-consulting.be
www.arche-consulting.be
Tel: + 32 9 216 70 34

TABLE OF CONTENTS

1. TECHNICAL ANNEX A: METHODOLOGY IDENTIFICATION AND UNDERSTANDING CO-EXPOSURE AND MIXTURE RISK IN THE ENVIRONMENT

This section outlines the methodology used for identification and understanding co-exposure and mixture risk in the environment. It includes a description of which databases have been considered, how the data have been processed and analysed.

1.1. DATABASES

1.1.1. OVERVIEW DATABASES

To collect environmental monitoring data, online databases were screened. The focus is on aquatic, dissolved exposure, though data for sediment and soil were also considered. Especially sediment data are relevant because sediment are sinks for many chemicals entering the aquatic environment and could therefore be more reflective of long-term averaged co-exposure. Also, several biodiversity indices are based on macro-invertebrates in/on the sediment. However, this compartment is harder to monitor and thus extensive monitoring databases were not found.

Following criteria were used to select suitable databases: (1) a wide range of chemicals were monitored, covering as many chemical groups (e.g. agrochemicals, industry chemicals, metals, pharmaceuticals) as possible; (2) samples are linked to a location and time (3) ideally, the biological status of the waters is also monitored.

In the project, three types of monitoring databases were considered: European-wide databases, river basin databases and national or regional databases. An overview of the databases considered in the project can be found in Table 1. Based on the final size of the datasets (see Section 1.2), data availability and homogenity and the spatiotemporal coverage, the following databases were chosen for further analysis: EEA Waterbase (referred to as “Waterbase”), Danube River Basin Water Quality Database (“Danube”), ICPR – Rhine water quality (“Rhine”), eauFrance, Flemish environmental agency (VMM) environmental quality database (“Flanders”) and Waterkwaliteitsportaal (“Netherlands”).
A critical note has to be made with regard to the use of classical regulatory monitoring campaigns (such as under the Water Framework Directive WFD). These, typically focus on a limited, predefined set of priority substances. E.g. the Flemish Environment Agency reported concentrations for 34 different organic substances ‘only’ with special focus on pesticides and PAHs, in addition to 22 metals. However, many of the inland waterways in Europe are also impacted by the presence of the new “Watch List” chemicals, emerging drugs and nutrients that are not currently regulated under the WFD. Water regulators and authorities do not always know the levels, locations or impacts of these pollutants. Indeed, scientific monitoring approaches highlighted the co-occurrence of hundreds of chemicals in different freshwater bodies (e.g. Loos et al., 2009, 2013; Moschet et al., 2014; Munz et al., 2017). Busch et al. (2016) described the diversity of potential molecular targets for contaminant-biosystem interactions: 426 organic chemicals were detected in three EU rivers, including 173 pesticides, 128 pharmaceuticals, 69 industrial chemicals and 56 other compounds.

Table 1: Databases considered in this project.

Type	Database	Description
European	EEA Waterbase	Status and quality of Europe's rivers, lakes, groundwater bodies and transitional, coastal and marine waters, on the quantity of Europe's water resources, and on the emissions to surface waters from point and diffuse sources of pollution. Link: https://www.eea.europa.eu/data-and-maps/data/waterbase-water-quality-icm-1
	IPChem	European Commission's reference access point for chemical occurrence data in Europe. Link: https://ipchem.jrc.ec.europa.eu/index.html#discovery
	NORMAN network	This EU network of laboratories and research centers in the field of monitoring emerging environmental substances curates a database that includes exposure, ecotoxicological and bioassay monitoring data. Link: https://www.norman-network.com/nds/
River basin	Danube River Basin Water Quality Database	Comparable and reliable information on water quality (chemical, biological and effect monitoring) for the whole length of the Danube River including the major tributaries. Link: https://www.icpdr.org/wq-db/
	ICPR – Rhine water quality	Good database with several sites across countries monitored over many years along the Rhine. Link: https://www.iksr.org/en/topics/water-quality/water-quality-data
National / Regional	eauFrance	Data of surface water in France https://geo.data.gouv.fr/fr/datasets/5a3bfb08ce37de663133c8c19034231b4fc6d76c http://www.naiades.eaufrance.fr/france-entiere#/
	Flemish environmental agency (VMM) environmental quality database	VMM has been monitoring water quality indices (chemical and biological status) in Flanders for decades, including species abundance and biodiversity indices[1]. http://geoloket.vmm.be/Geoviews/
	HMS (UK)	Historic UK Water Quality Sampling Harmonised Monitoring Scheme Summary Data https://data.gov.uk/dataset/bda4e065-41e5-4b78-b405-41c1d3606225/historic-uk-water-quality-sampling-harmonised-monitoring-scheme-summary-data
	Swedish University of Agricultural Science (SW)	Swedish national data hosts for data collected from national and regional fresh water monitoring, as well as from recipient monitoring (chemical & biological monitoring in Sweden). http://www.slu.se/miljodata-MVM

[1] Viaene et al 2017. Multivariate analyse van VMM meetdata met behulp van ordinatietechnieken en drempel-waardeanalyse. Bestek nr. A 2017 S 0001 L.

		https://www.slu.se/en/departments/aquatic-sciences-assessment/
	Waterkwaliteitsportaal (NL)	Waterkwaliteitsportaal collects data for the WFD (surfacewater & groundwater) https://www.waterkwaliteitsportaal.nl/wkp.webapplication

1.1.1.1. WATERBASE

Waterbase is the generic name given to the EEA's databases (European Environment Agency) on the status and quality of Europe's rivers, lakes, groundwater bodies and transitional, coastal and marine waters, on the quantity of Europe's water resources, and on the emissions to surface waters from point and diffuse sources of pollution. The dataset contains time series of nutrients, organic matter, hazardous substances and other chemicals in rivers, lakes, groundwater, transitional, coastal and marine waters. The data has been compiled and processed by EEA.

For this project, the database on chemical status and quality was used (Waterbase quality ICM). Specifically, we used the dataset T_WISE6_DisaggregatedData containing the disaggregated water quality data on the observed values (e.g. concentrations) of determinants in water, sediment and biota samples in inland, coastal and marine waters as reported by EEA Member Countries on an annual basis.

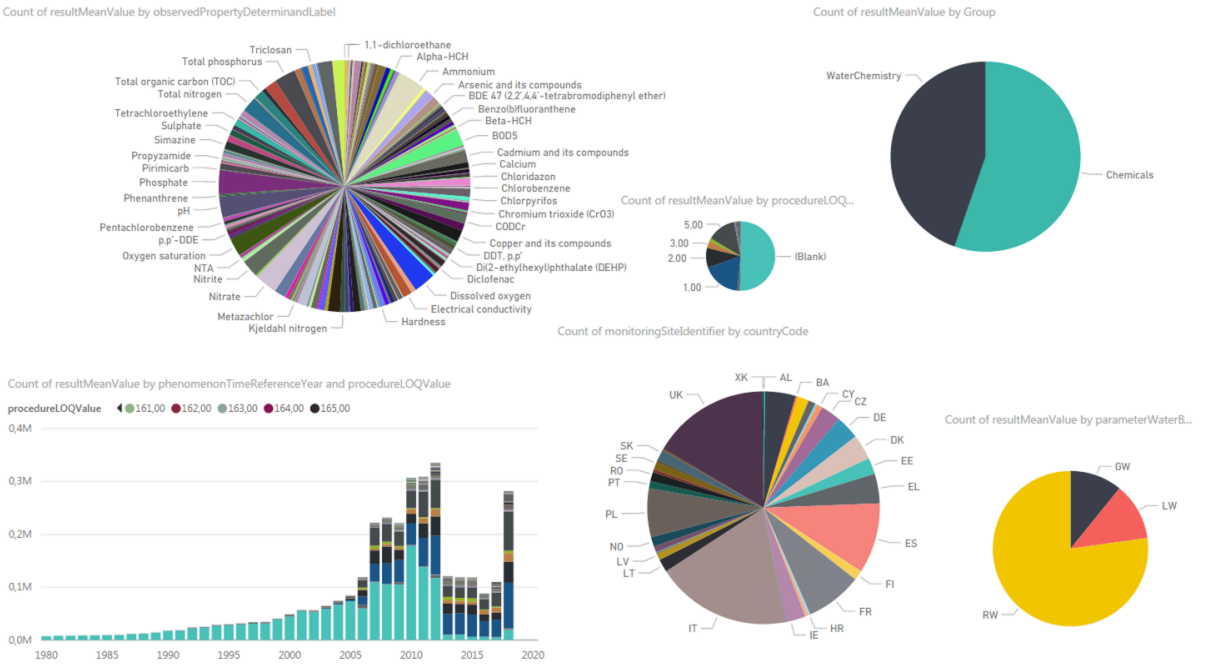


Figure 1: Infogram on Waterbase data set (top left: number of samples per parameter, top right: number of sample per parameter group, bottom left: number of samples versus time, bottom centre: number of samples per country, bottom right: number of samples per type of water body (RW=river water; GW=ground water; LW=lacustrine water), central: number of samples detected (blank) and non-detected with the various LOQ.

Additionally, data on the biological status of the waters (Waterbase - Biology) was also gathered. The dataset contains time series of data on biological quality elements (BQEs) such as phytobenthos and macroinvertebrates in rivers, lakes, transitional and coastal waters.

This is a very extensive European dataset, covering a wide range of chemicals (Figure 1). Member states report at least the priority pollutants. However, the complete range of chemicals in the dataset is much wider (449 different chemicals). For most samples, extensive information on the space and time of the sampling is available. The availability of biological monitoring data is an additional strength of this database. This database was considered **useful** for the aims of the current project.

1.1.1.2. NORMAN

The NORMAN network enhances the exchange of information on emerging environmental substances, and encourages the validation and harmonisation of common measurement methods and monitoring tools so that the requirements of risk assessors and risk managers can be better met. NORMAN organises the development and maintenance of various web-based databases for the collection & evaluation of data / information on emerging substances in the environment For the current project, the Substance database and the Chemical Occurrence database were most useful. The Substance database is a merged list of NORMAN substances, offering a central database with various substances for suspect screening and prioritization. The Chemical Occurrence Data is a database of geo-referenced monitoring data on emerging substances. The number of samples in the database are quite limited (<1000). Measurements from Phenanthrene in Slovakia and 2008 are dominating the databases (see Figure 2). Chemicals in the NORMAN database are typically measured individually and not as part of a mixture. Therefore, the dataset to analyse for mixture data is **too limited** to take into account for analyses.

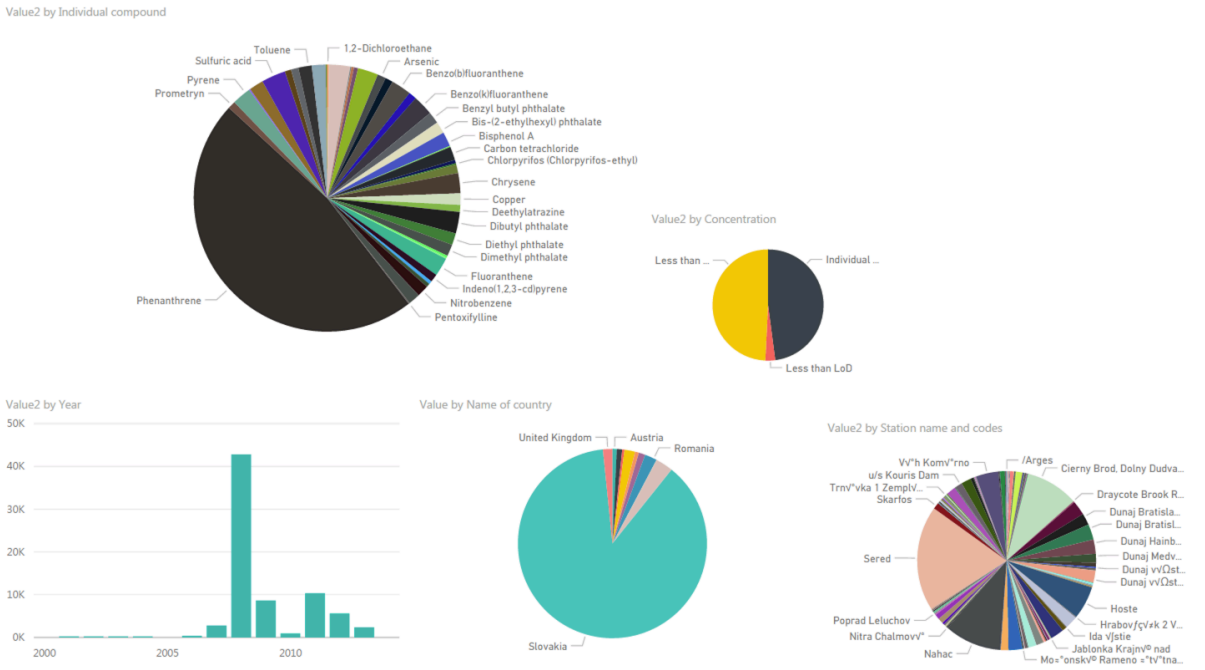


Figure 2: Infogram on NORMAN data set (top left: number of samples per parameter, bottom left: number of samples versus time, bottom centre: number of samples per country, bottom right: number of samples per river, central: number of samples detected (blank) and non-detected.

1.1.1.3. IPCHEM

The Information Platform for Chemical Monitoring (IPCHEM) is the reference access point for discovering chemical monitoring data collections which are managed by and are available to European Commission bodies, Member States, international and national organisations and research communities. The Platform aims to support a more coordinated approach for collecting, storing, accessing and assessing data related to the occurrence of chemicals and chemical mixtures, in relation to humans and the environment. IPCHEM is designed and implemented as a de-centralised system, providing remote access to existing information systems and data providers.

At the time of project initiation and data gathering, the IPCHEM database portal was being revised. Data selection was possible on individual chemicals or for all chemicals measured in a geographical area. The latter was still in beta and the number of samples accessible this way was limited. Additionally, the IPCHEM database is a collection of individual databases that not necessarily cover the same chemicals. Therefore, the choice was made to focus on other databases.

1.1.1.4. DANUBE BASIN

The International Commission for the Protection of the Danube River (ICPDR) works to ensure the sustainable and equitable use of waters in the Danube River Basin. The ICPDR addresses the entire Danube River basin, comprising 19 countries, making it the most international river basin in the world. Including more than 300 tributaries and connected groundwater resources too, this makes the ICPDR one of the largest and most active international river basin management commissions in the world. The river basin covers 817,000 square kilometers and 83 million people live in its catchment area. The Danube passes through numerous large cities – including four national capitals, Vienna, Bratislava, Budapest and Belgrade.

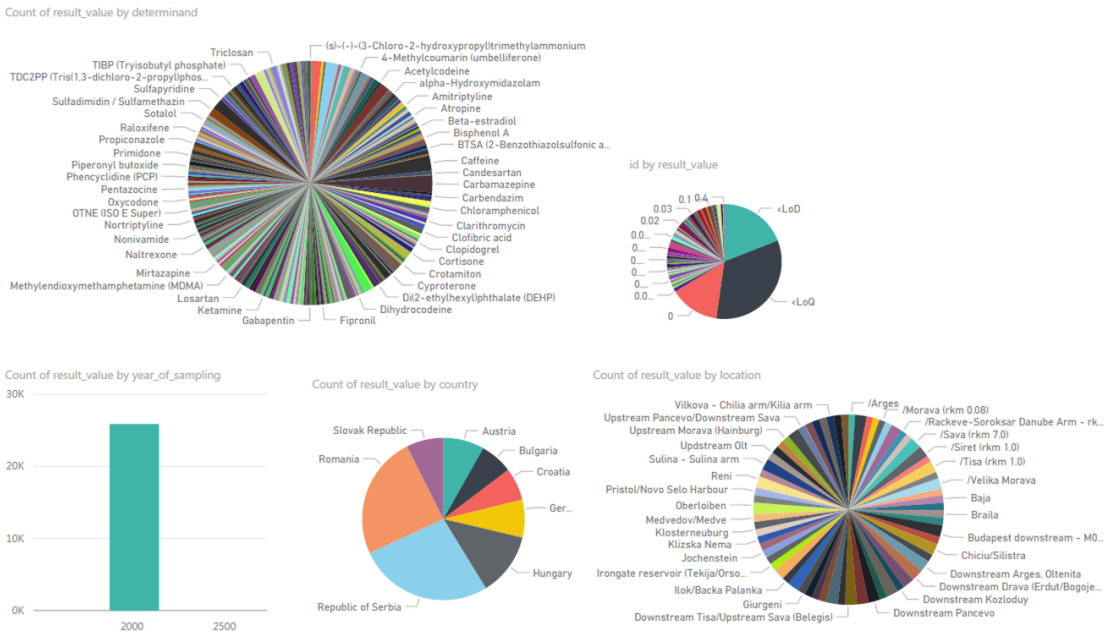


Figure 3: Infogram on Danube data set (top left: number of samples per parameter, bottom left: number of samples versus time, bottom centre: number of samples per country, bottom right: number of samples per river, central: number of samples detected (blank) and non-detected.

A Joint Danube Survey is carried out only once every six years – JDS1 was held in 2001 and JDS2 in 2007. JDS3 completed the sampling in 2013 to enter an extensive analysis stage and published the final reports in 2015. The Joint Danube Survey 3 (JDS3) was the world's biggest river research expedition of its kind in 2013, the UN International Year of Water Cooperation. For six weeks between 13 August and 26 September 2013, the JDS3 ships travel 2,375 km downstream the Danube River, through 10 countries, to the Danube Delta.

While the JDS3 campaign covered a large geographical area, the temporal dimension was limited. This resulted in a database with a limited number of locations/samples (40). The chemical monitoring for chemicals was very extensive, with 395 chemicals monitored in total. This included traditional substances (pesticides, priority pollutants) as well as emerging pollutants e.g. pharmaceuticals. This feature of the database hinders the planned statistical analyses (PCA) of the database. For the analyses in this project, the JDS3 database is thus **not useful**.

1.1.1.5. RHINE BASIN

The state of the Rhine is being monitored from Switzerland until the Netherlands by ICPR – International Commission for the Protection of the Rhine. The first monitoring was carried out as early as the 1950ies, so that, for certain substances in the Rhine, corresponding time series are available. Over time, more and more substances were included in monitoring activities, the measurement of suspended matter was expanded and the sampling frequency was increased. Monitoring results are supplemented every year, thus continuing time series (see Figure 4). The data can be downloaded at https://www.iksr.org/en/topics/water-quality/water-quality-data.

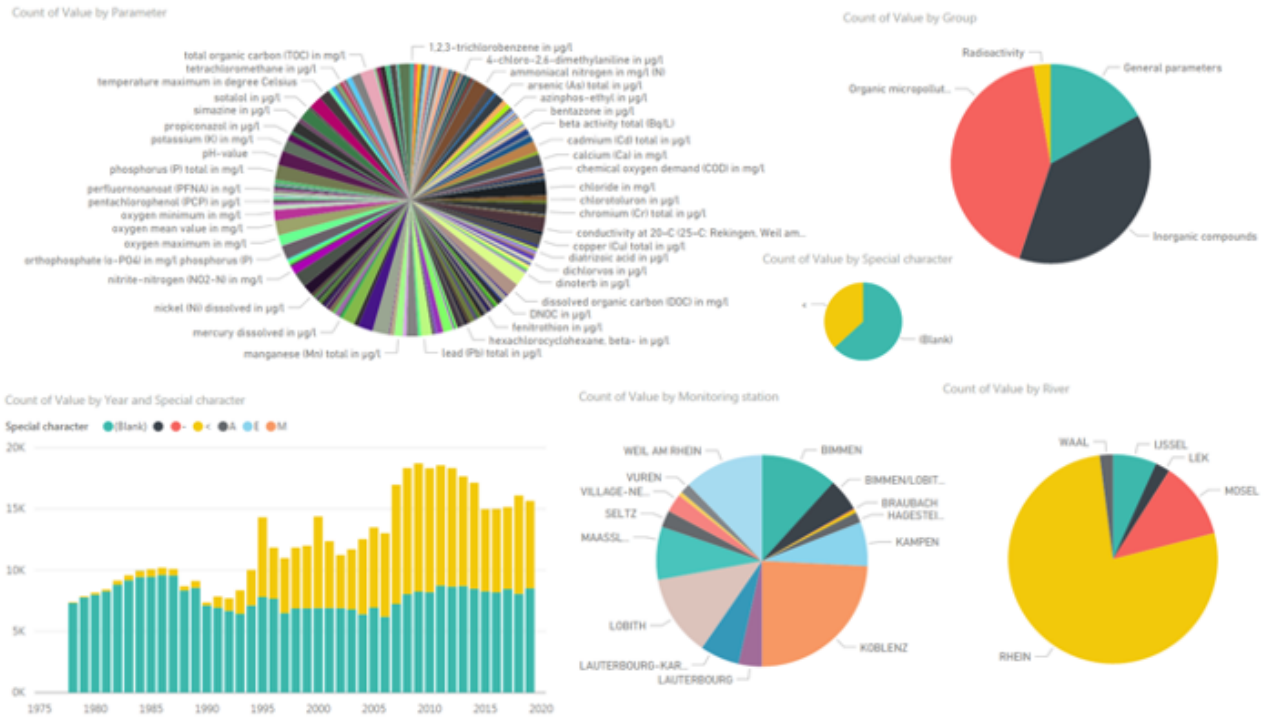


Figure 4: Infogram on Rhine data set (top left: number of samples per parameter, top right: number of sample per parameter group, bottom left: number of samples versus time, bottom centre: number of samples per monitoring location, bottom right: number of samples per river, central: number of samples detected (blank) and non-detected ("<")).

The monitored parameters include both organic micropollutants, inorganic compounds and general water quality parameters. The monitored chemicals are mainly priority substances under the EU Water Framework Directive. The sampling intensity has increased over time. The monitoring locations are mainly located at the river Rhine and some major tributaries. Therefore, this data set does not monitor and is not representative for the entire catchment. Also, the number of monitoring locations is rather small. Only about 6 monitoring locations have been intensively monitored. The database is nevertheless considered as a **good database** because it includes many samples in time (on the same location) allowing to assess temporal patterns in co-exposure.

1.1.1.6. FRANCE (EAUFRANCE)

In France, there are 6 water agencies established since 1964 consisting of 6 big water basins Adour-Garonne, Loire-Bretagne, Seine-Normandie, Artois-Picardie, Rhin-Meuse and Rhône-Méditerranée. These water agencies carry out a mission for general interest, including managing, preserving water sources and aquatic environment. The analysis on water quality has been started by these agencies ever since. The data is annually updated in the database of eauFrance and can be downloaded at http://www.naiades.eaufrance.fr/.

In scope of this study, two datasets of EauFrance were selected: a local dataset of Adour-Garonne of period 2015-2019 and a dataset of entire France of period 2014-2019.

Adour-Garonne dataset:

This dataset can be downloaded separately at this link. The measurement was started in 1971 and has been regularly updated. For the current study, period 2015-2019 was selected. The data package includes physchem, phytosanitary and hydrobiology data (see Figure 5).

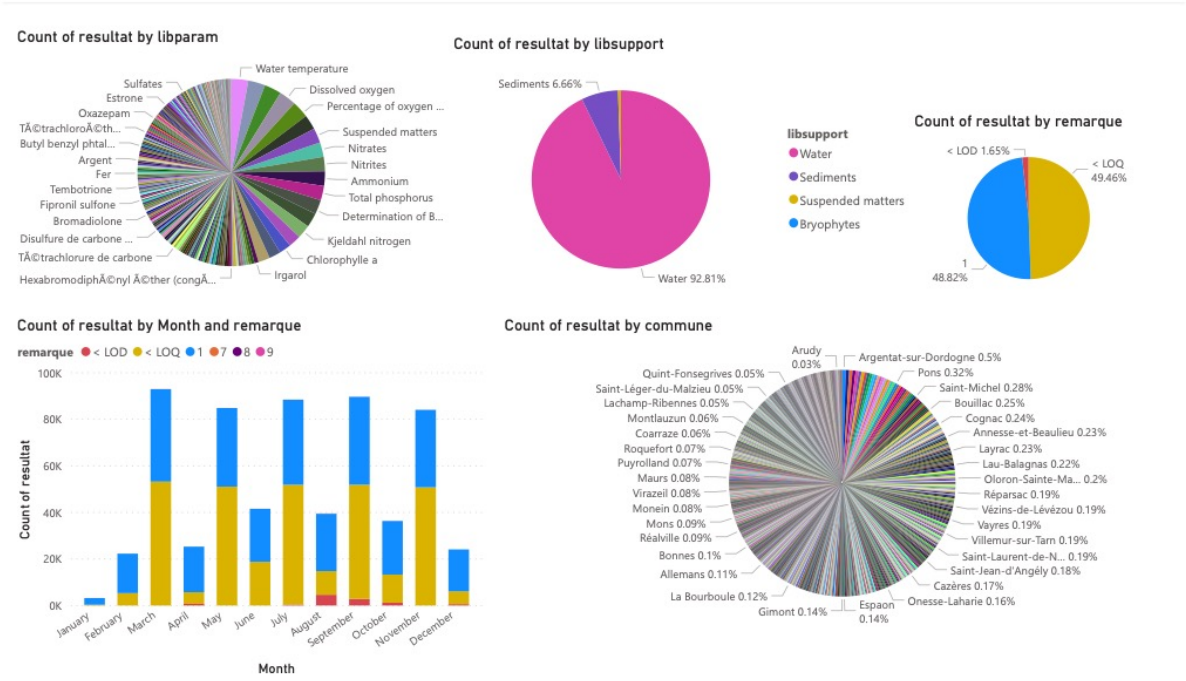


Figure 5: Infogram on Adour-Garonne data set (top left: number of samples per parameter, top center: number of sample per compartment, top right: number of samples detected ("1") and non-detected ("<LOQ"), bottom left: number of samples versus time, bottom right: number of samples per monitoring location.

The physchem data includes more than 2000 sampling sites covering 7 sub basins in the region, with more than 409 variables. The data consist of 38.9% of water quality parameters (eg: pH, temperature, dissolved oxygen, etc.) and the rest is chemical substance measurement. Since water quality parameters are not relevant for this study, the focus is set on chemical substances which are consisting of 47% industrial substances, 12% agrochemical substances, 21% pharmaceutical substance, 5% PAHs and 15% of mixed-use substance. With the diversity of measured chemicals, large number of sampling sites and broad period of time, this dataset is considered as a **good database** to further investigate in this project.

Entire France dataset:

For the current study, the period 2014-2019 was selected. The data package includes physchem, phytosanitary and hydrobiology data. A total of 1880 chemicals were reported in the dataset over 12235 sites. This means that this database was used as a **good database** in the current study.

1.1.1.7. FLANDERS (VMM)

Flanders Environment Agency (abbreviated VMM – Vlaamse Milieu Maatschappij) is an agency of the Flemish government working towards a better environment in Flanders. In

addition to its other tasks, VMM measures and controls the quantity and the quality of surface water, groundwater and sediments and reports about the results. This covers all main rivers in Flanders (Maas, Scheldt, Yser) and its tributaries. VMM monitors both chemical and biological water quality.

The data from 2007-2015 were available for use in this project. Both inorganics (metals) and organic pollution (12 PAHs and 22 pesticides) were monitored. There is a good spatial and temporal coverage of the database, and a good mix of organic and inorganic pollution. Additionally, the biological status linked with the chemical status enables an impact analysis of the mixture exposure. Therefore, this was considered a **useful database** for this analysis.

1.1.1.8. THE NETHERLANDS

"Het Waterkwaliteitsportaal" (WKP) collects and curates the for the Water Framework Directive. This allows to monitor the water quality in The Netherlands. Additionally, the data from the annual Landelijke Enquete Waterkwaliteit is accessible through the WKP. The database can be accessed here: https://www.waterkwaliteitsportaal.nl/wkp.webapplication.
Another Dutch database is "De Bestrijdingsmiddelenatlas". This database is focused on pesticides in the surface water. It covers up to 880 chemicals throughout The Netherlands. The data can be found here: https://www.bestrijdingsmiddelenatlas.nl/atlas/1/1

Both of these are **good resources** for this project, with the limitation that they are focused on PPP (plant protection products). Therefore, their use for assessing mixture toxicity outside of PPP regulations is more limited.

1.1.1.9. SWEDEN

The Swedish databases are managed by the Swedish Agency for Marine and Water Management. In scope of this project, two datasets, which were generated from two separated monitoring programs, are considered: (i) Environmental data for lakes and water courses and (ii) Data of pesticides in surface water

(i) Environmental data for lakes and water courses:
This data has been collected since 1941. In the most recent data (2020), more than 4500 sampling sites were taken acrossing Sweden with 103 variables. However, the monitoring program is only limited to water quality parameters and cations/anions. Thus, the dataset is considered not suitable to be further investigated in scope of this project.

(ii) Data of pesticides in surface water:
This dataset consists of monitoring data of plant protection products in surface water from four small agriculturally dominated catchments and two rivers in the south of Sweden from 2002 to 2019. There are 176 PPP substances measured in this program. Even though the dataset has broad timeline and extending list of chemicals, this dataset is considered not suitable to be further investigated in this project due to its limited number of sampling sites.

1.1.1.10. UK

The Historic UK Water Quality Sampling Harmonised Monitering Scheme (HSC) dataset was started in 1974. The dataset is large and includes 230 sampling sites all over UK. Despite the extensiveness of the dataset, most parameters covered in the scheme are water quality parameters (eg. dissolved oxygen, nitrates, orthophosphates) and metals. Only a few agrochemicals (7), industrial (4) and 2 PAHs substances are also covered in this monitoring program. Considering the limited number of substances measured, the UK's dataset is considered not suitable to be further investigated in this project.

1.2. DATA PROCESSING

1.2.1. DATA SELECTION

Several databases do not have a broad and complete set of monitored chemicals which limits co-exposure assessment. The databases are often collections of monitoring data taken by different entities which each have their own protocols and objectives. Understanding co-exposure and multivariate statistical techniques assessing co-occurrence require a **complete dataset** i.e. for each row in the dataset (observation), all columns (variables) should contain values (detected or non-detected). For the chemical monitoring databases, this means that for each sampling time and location, a measurement should be available for each chemical in the dataset. The selected databases contain measurements for many chemicals (often 100+), but chemicals are often only measured in a subset of samples, and these subsets of samples do not necessarily overlap. **Data selection** is thus needed to maximize the number of locations and sampling times while retaining a high number of chemicals. An algorithm was used that iterates removal of chemicals with the lowest number of measurements and removal of samples with the lowest number of observedchemicals, until a complete dataset is retained.

This data selection processing step greatly **reduced the size of the datasets** (see Table 2During data processing, a step is sometimes needed to **summarize/aggregate the data**, for a multitude of reasons: chemicals are not necessarily sampled on exactly the same date or on the same location; multiple samples were taken on the same data or multiple measurements were performed; the temporal resolution is not as required e.g. weekly while monthly measurements are needed; etc. By smart aggregation, the number of rows (locations and dates) and columns (chemicals) can be maximized. The ideal aggregation option will depend on the original database and is discussed for each database separately below.

) but allows to observe co-exposure in mixtures and perform the selected multivariate techniques. The data points lost are the locations where only a small number of chemicals were measured or the chemicals that were measured in a limited number of locations. This means there is a bias in the processed datasets for samples with many chemicals measured simultaneously. Mixtures of smaller number of chemicals might be missed, however, following this approach.

During data processing, a step is sometimes needed to **summarize/aggregate the data**, for a multitude of reasons: chemicals are not necessarily sampled on exactly the same date or on the same location; multiple samples were taken on the same data or multiple measurements were performed; the temporal resolution is not as required e.g. weekly while monthly measurements are needed; etc. By smart aggregation, the number of rows (locations and dates) and columns (chemicals) can be maximized. The ideal aggregation option will depend on the original database and is discussed for each database separately below.

Table 2: Sizes of the databases before and after data selection. The number of observations can be interpreted as the number of measured mixtures with mixture size equal to the number of chemicals.

	Before data selection		After data selection	
Database	**Number of observations**	**Number of chemicals**	**Number of observations**	**Number of chemicals**
Waterbase	579,882	452	27,666	76
NORMAN	1,040	339	66 sites	19
Rhine	840 (16 sites)	228	390 (8 sites)	71
Danube	191	395	44	248
EauFrance-Garonne	87721	401	3493	77
Flanders	9,338	186	1,553	44
UK	205,479	83	9,134	44

When aggregating multiple measurements to one value, there are several options e.g. taking the mean of all measurements, the maximum, the 10^{th}- or 90^{th} percentile. Where needed, we chose to take the maximum of the measurements being aggregated: aggregation was typically done per month per location. By taking the maximum, we avoid issues with how to deal with detection limits when aggregating the data (see next section) e.g. how to take the average of three samples if two are below the detection limit.

Finally, before analysis, the chemical concentrations were **log-transformed** ($\log(x+10^{-5})$ to be precise), to avoid issues where concentrations of 0 were reported). Additionally, the concentrations were **standardized** by subtracting the values by the mean (centering) and dividing by the standard deviation (scaling). This allowed easier comparison between chemicals which can have up to orders of magnitude differences in concentration.

1.2.2. DEALING WITH DETECTION LIMITS

It is more difficult to analyse the databases if a monitored chemical is not detected, especially when the majority of the samples are not detected. Such a result does not prove that the compound is not present or absent, it only shows that the concentration is somewhere between zero and the LOD (Limit of Detection).

Dealing with detection limits for characterising co-exposure

For the purpose of identifying and characterizing co-exposure, the issue of detection limits is somewhat circumvented since the absolute values of the environmental concentrations are less relevant (the values are transformed anyway). Only the relative values of environmental concentrations/detection limits are relevant because the main purpose of correlation analysis and principal component analysis is to identify relationship between variables, in this case to identify which chemicals co-occur in mixtures and which do not. For the purpose of the calculation, the detection limit was assumed as the observed concentration for all non-detects. This is not impacting the conclusions on co-exposure and identification of mixtures.

Dealing with detection limits for calculation of cumulative risk

For the purpose of calculating cumulative risk, any assumption on the non-detects has an implication on the calculated cumulative risk and, therefore, this needs to be further considered.

Assuming a zero concentration for all non-detects will therefore underestimate the total risk, if no additional knowledge about e.g. emission or use pattern is available (Gustavson et al., 2017). On the other hand, assuming that all non-detected compounds are present just below their LOD – the worst-case scenario that is still compatible with the recorded values – is also unrealistic. Such an approach immediately leads to the logical inconsistency that the estimated risk becomes simply dependent on the number of compounds analyzed. The same is true for setting the concentration used for the risk assessment a priori to any other value above zero (Gustavson et al., 2017). To further demonstrate this point, an example calculation is made as follows: assume a river sample of pristine origin (i.e. without any contaminants). The hazard index (or cumulative risk) is zero. Assume that chemical analysis is conducted for multiple contaminants, each will result in a non-detect. Setting the non-detects at the detection limit or any other value below the detection limit will generate a calculated artificial toxic pressure whereas the reality is there no toxic pressure. In Figure 6, the cumulative risk of the non-detects shows that substantial mixture pressure is already detected when the non-detects are present at the detection-limit both for the Adour-Garonne dataset and the Rhine dataset. Most of the mixture exposures are categorized under mixture Group I, which indicates that for these mixture exposures the detection limit of at least one of the substances is higher than the selected ecotox reference value (HC_5). Price & Han (2011) also demonstrated higher hazard indices when detection limits are included in the mixture assessment. This analysis clearly shows that there is an issue with non-detects values when assessing cumulative risks for complex databases. In addition, it also clearly shows that the environmental threshold level should be taken into account when selecting chemical measurement techniques and its associated detection limit.

Parametric and non-parametric statistical methods are available for data with “less-than” values, i.e. findings of concentrations < LOD. They allow the estimation of the likely contribution of non-detects to the total risk quotient. These methods typically can deal with non-detects as visualized in Figure 7 right. Parametric methods are largely not applicable since the distributional assumptions of parametric methods cannot be verified due to the higher number of non-detects (as visualized in Figure 7 left). Non-parametric methods avoid a distributional assumption but are nevertheless subject to empirical assumptions which require a minimum amount of detected values in order to provide robust results. These are not able to deal with high number of non-detects as in Figure 7 left. Figure 7 left describes a situation with so-called zero-inflation phenomenon, a data set with a lot of zero or close to zero values combined with a high detection limit: 80% non-detects can mean 20%<DL and 60% absent.

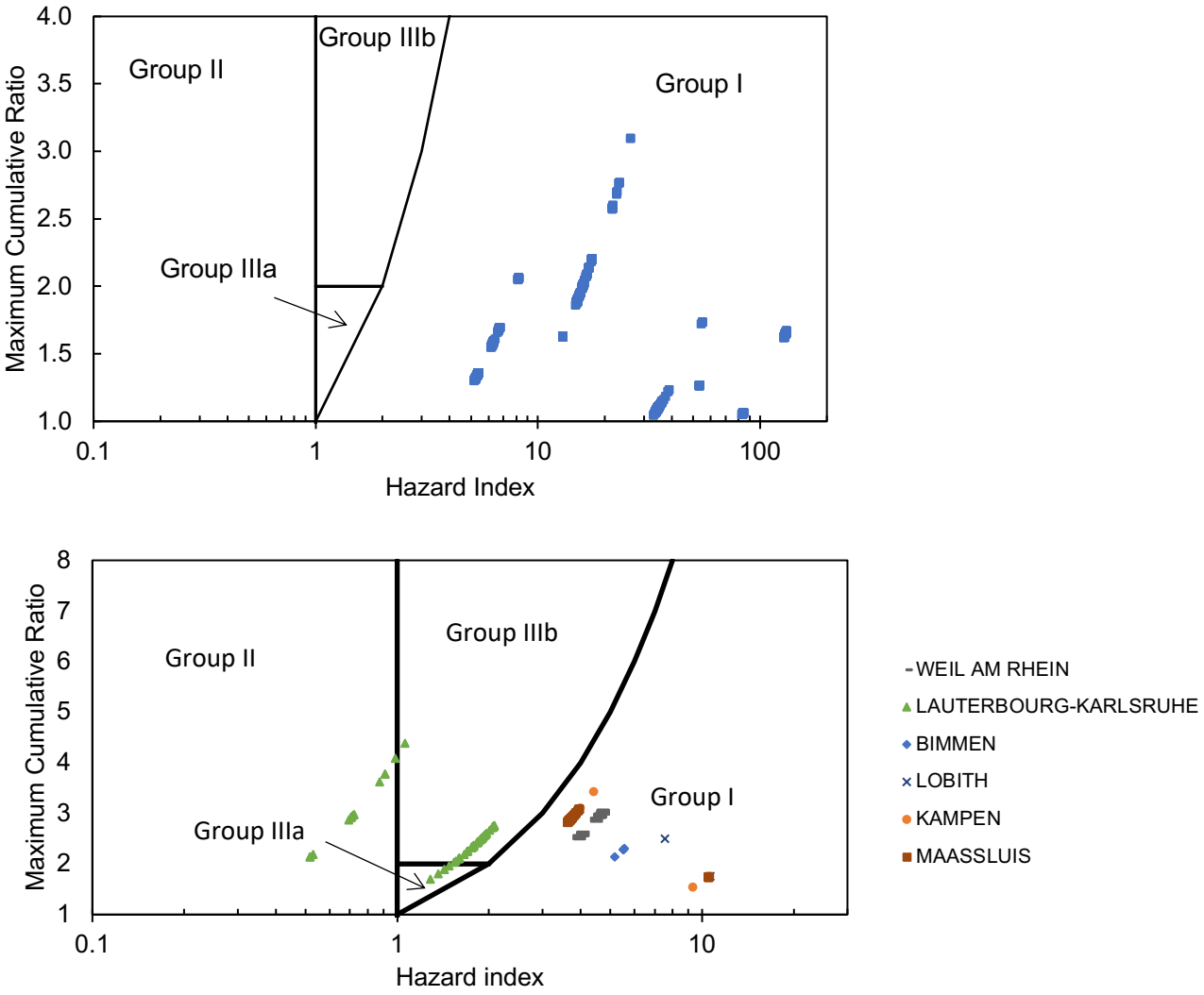


Figure 6: Plot showing the Maximum Cumulative Ratio as a function of the Hazard Index for the Adour-Garonne dataset (upper panel) and the Rhine dataset. Cumulative risks are shown only for the non-detects at their detection limit. (<DL=DL and >DL=0)

A binary transformation can be applied in case of high number of non-detects. Here, the non-detects are replaced by 0 and the detected concentrations are replaced by 1. Quantitative information on the concentration is lost in this transformation. This makes it less suitable for calculating toxic units.

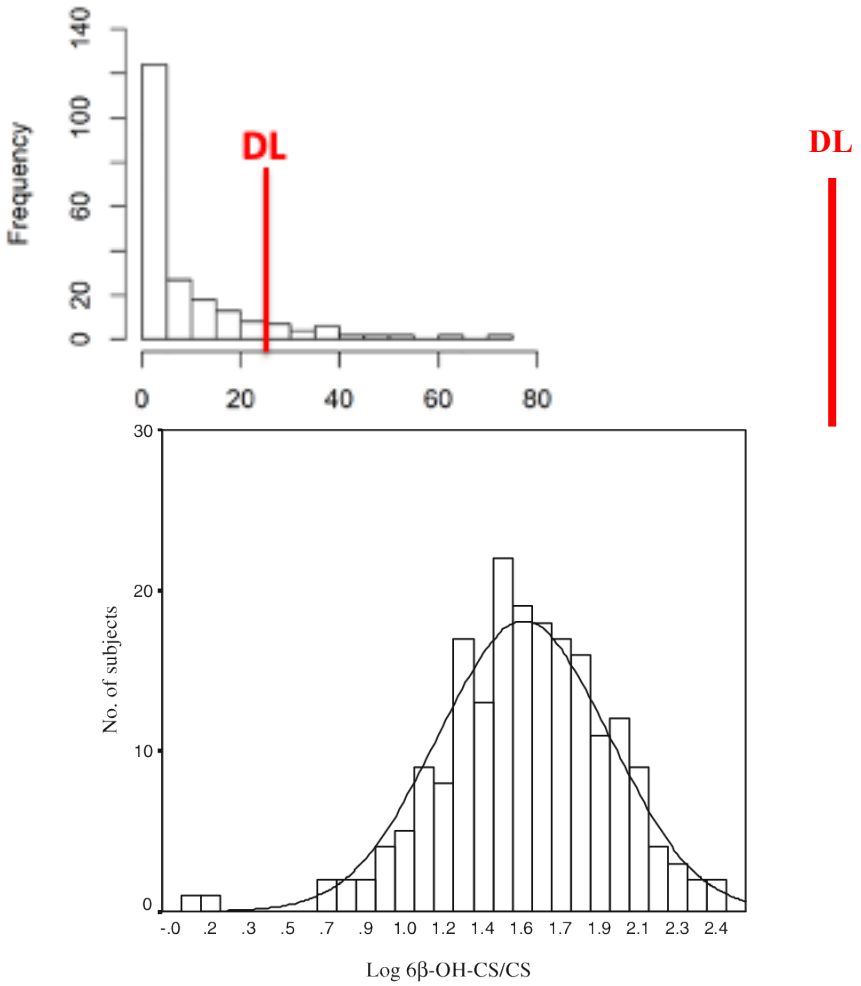


Figure 7: Examples of frequency distributions: left a data set with a lot of zero or close to zero value combined with a high detection limit versus right a dataset combined with lower detection limit

In summary, many of the available statistical techniques dealing with "less-than" values are not fit for data set with >50% of non-detected values. As a result, sensitivity analysis will be carried out using following two scenarios and considering the first scenario being more relevant:

- Assuming zero concentration for all non-detects which is conceptually a best-case scenario but is probably closer to reality than the next scenario because of 1) the high number of non-detects (and potential the zero-inflation phenomenon) especially when expressed as hazard quotients or risk ratios and 2) in general, mixture interactions (including antagonism, potentiation, and synergies) usually occur at medium or high dose levels (relative to the lowest effect levels). At low exposure levels, they are either unlikely to occur or are toxicologically insignificant (SCHER, 2011). And 3) it is widely described in literature that typically only a handful substances contribute to the mixture hazard index or cumulative risk.
- Assuming detection limit as the observed concentration for all non-detects, which is conceptually a worst-case scenario but is most likely unrealistic, especially when detection limit is close to the HC5 or PNEC.

1.3. DATA ANALYSIS

1.3.1. PRINCIPAL COMPONENTS ANALYSIS (PCA)

1.3.1.1. BACKGROUND OF THE TECHNIQUE

Principal components analysis (PCA) is a method to summarise, in a low-dimensional space, the variance in a multivariate scatter of points. In doing so, it provides an overview of linear relationships between objects and variables. This can often act as a good starting point in multivariate data analysis by allowing you to note trends, groupings, key variables, and potential outliers.

The objective of a principal component analysis (PCA) is to determine the correlations between parameters in a dataset. Through data reduction, primary components (PC) are constructed by combining closely correlated parameters. Principal components are calculated from the eigenvectors of the covariance matrix of the data. The eigenvectors are the principal components of the ellipsoid determined by the covariance matrix (more or less the point cloud of the data). One requirement is that parameters should be linearly correlated. If not the case, this can be attained by performing data transformations, typically log transformations.

To better interpret correlations between parameters, the principal components can be rotated using the technique called **varimax rotation**. The varimax rotations make it easier to attribute a given variable to a single component and the overall interpretability of the PCA may be simplified. This allows to select sets of variables that group together and will be used to identify common mixtures of chemicals.

1.3.1.2. VISUALISATION WITH BIPLOTS

The results of a PCA analyses are typically visualised using a biplot (Figure 8).

- Distances between object points approximate the Euclidean distances between objects. Thus, objects ordinated closer together can be expected to have similar variable values.
- Right-angled projections of an object point on a variable's vector (arrow) approximates the value of that variable for the chosen object
- The length of a variable vector in the ordination plot reflects its contribution to the ordination. That is, variables with vectors which appear longer than others in a given ordination were more important in building the PCs (principal components) used in that ordination. The contribution of a variable to a particular PC can be approximated by projecting the "tip" of the vector onto the PC of interest.
- Angles between variable vectors are meaningless

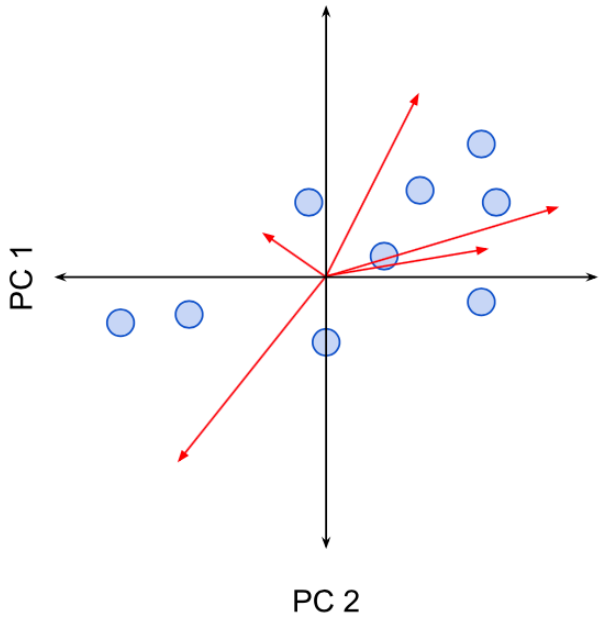


Figure 8: A PCA biplot. Points represent objects (rows). Red vectors represent the original variables (columns) used to build the PCs. *Source: https://mb3is.megx.net/gustame/indirect-gradient-analysis/pca.*

1.3.1.3. CO-EXPOSURE OF CHEMICALS WITH PCA

Within this project, PCA was used to

- Identify common and rare groups of sites with co-exposure as well as combinations of chemicals that do not co-exist (based on the position and the distance between points (sites)). Clustering of sites indicate sites that have a similar exposure profile and are thus interesting to group for further analyses.
- Identify sites with high ("hot spots") or low observed exposure concentration for the pre-dominating multiple chemicals (based the position of the points to the axis).
- Assess correlation or absence of correlation (independence) between chemicals based on the length of the arrows and their angle.

The PCA analysis will identify sites with similar exposure sites as well as identify the substances that are most correlated with the variation in the datasets. This will help prioritize which sites and most likely combinations of mixtures of mixtures of concern should be prioritized on in the subsequent analyses.

Rather than assessing individual chemicals, it is also proposed to assess use categories and/or chemical group (such as done in Busch et al., 2016 as shown in Figure 9) in relation to co-exposure. This allows to simply the analysis of multiple chemicals at the same time and to cover relevant unmeasured industrial chemicals and pharmaceuticals.

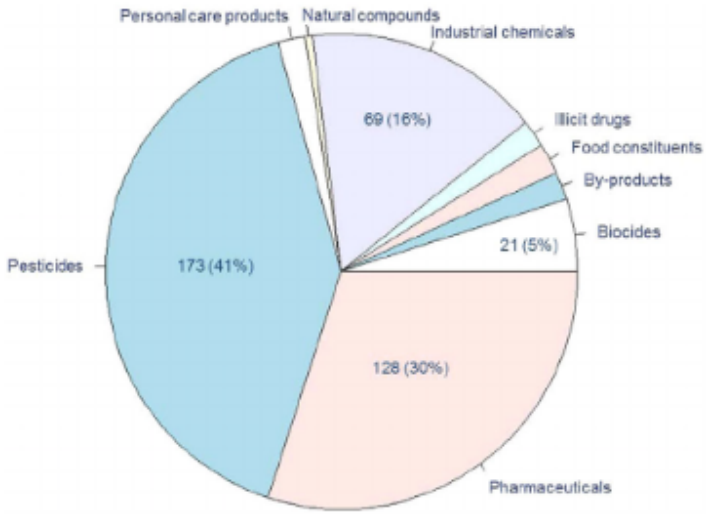


Figure 9: Use categories of organic compounds detected in European rivers (Busch et al. 2016).

1.3.2. UNDERSTANDING CO-EXPOSURE PATTERNS

From the data analyses, (groups of) sites (hotspots) will be identified that are most similar and (groups of) substances that are strongly correlated. Based on further exploration of these sites and substances, common patterns will be investigated. Options include:

- Characteristics of the clustered sites: are they situated on the same river? With the same/similar river characteristics (e.g. river size, stream velocity)? Are they clustered geographically (Country? Region?), …
- Applications and use of the selected chemicals. Are they agrochemicals, industrial chemicals, pharmaceuticals, household chemicals, …? Typical industries they are used in? Figure 1, for example, shows that the co-exposure of chemicals exists for PAH, as well as for herbicides. The former can be linked to an emission pattern from households and industry whereas the latter can be linked to an emission pattern of agricultural activities.
- Short-term use (periodical applications) or long-term use?
- Are they in the same chemical class?

Next, the common use patterns have been assessed for the identified typical mixtures. Several hypotheses can be put forward: typical mixtures or **co-occurrence of chemicals can be explained** by (see Figure 10):

- The **use pattern** (sources of household, industry, agricultural use), e.g., presence of pharmaceuticals is likely long-term use, and would be found downstream of WWTP (wastewater treatment plant), e.g., some plant protection products would be found near agricultural operations. The use pattern is also related to the chemical legislation (REACH, PPP, pharmaceuticals, etc…)
- **Spatial factors**, e.g., upstream river catchments would result in less complex mixtures, downstream river catchments result in more complex mixtures both in terms of number of chemicals as well as different type of chemicals/sources/use pattern
- **Temporal factors**, e.g., some agrochemicals may only be present seasonally.

Whilst all hypotheses will likely have impact on the co-occurrence of chemicals, analysis of the various data sets demonstrated that typical mixtures are mostly explained by their use pattern and spatial factors.

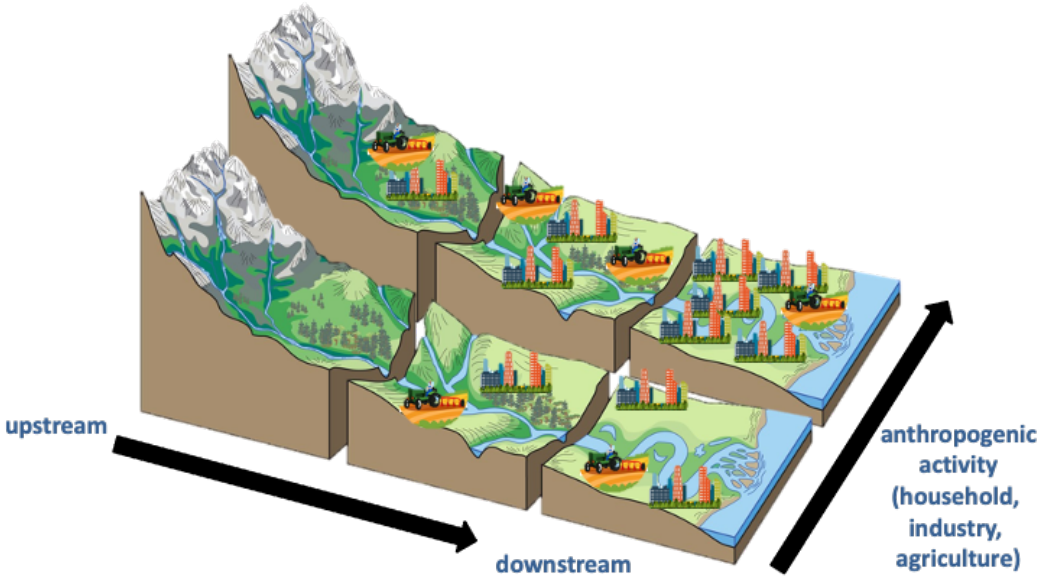


Figure 10: Chemical co-occurrence depends on chemical use pattern, spatial and temporal factors.

1.3.3. REDUNDANCY ANALYSIS (RDA)

Redundancy analysis (RDA) is a method to extract and summarise the variation in a set of response variables (here: biological impact indices) that can be explained by a set of explanatory variables (here: chemicals and other water quality indices). More accurately, RDA is a direct gradient analysis technique which summarises linear relationships between components of response variables that are "redundant" with (i.e. "explained" by) a set of explanatory variables. RDA can also be considered a constrained version of principal components analysis (PCA), wherein canonical axes - built from linear combinations of response variables - must also be linear combinations of the explanatory variables. The RDA approach generates one ordination in the space defined by the matrix of response variables and another in the space defined by the matrix of explanatory variables.

RDA ordinations may be presented as a biplot or triplot (Figure 11).

- Ojects ordinated closer together can be expected to have similar variable values. This will not always hold true, as RDA only recovers part of the variation in the data set.
- Right-angled projections of object points onto vectors (arrows) representing response variables approximate variable values for a given object.
- The angles between vectors representing response variables are meaningless.
- The angles between vectors representing response variables and those representing explanatory variables reflect their (linear) correlation.
- The length of vectors reflects their correlation with a given RDA-component. Long vectors nearly parallel to the axes reflect strongly correlated variables, short vectors indicate low correlations.

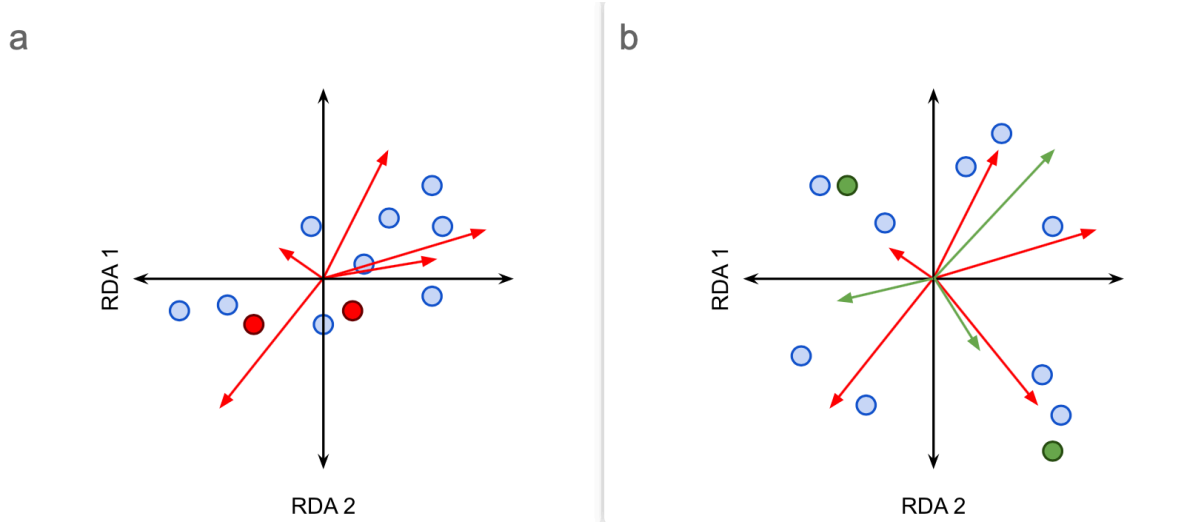


Figure 11: Schematic representation of a) an RDA biplot and b) an RDA triplot. a) An RDA biplot ordinates objects as points and either response or explanatory variables as vectors (red arrows). Levels of nominal variables are plotted as points (red). b) In a triplot, objects are ordinated as points (blue) while both response and explanatory variables (red and green arrows resp.) are plotted as vectors. Levels of nominal variables are plotted as points (green). Source: https://mb3is.megx.net/gustame/constrained-analyses/rda

1.3.4. GENERALIZED LINEAR MODELS (GLM)

Generalized Linear Models (GLMs) are a more general form of traditional linear models (regressions) with the advantage that the error structure does not have to be normally distributed. In GLMs, the value of a response variable can be expressed as a combination of predictor variables using a linear equation:

$$response = intersection + \beta_1 \cdot predictor1 + \beta_2 \cdot predictor2 + \varepsilon$$

The value of the respose variable is determined by the sum of the intersection (response value when all predictor variables are zero), the values of predictor variables multiplied by their respective coefficients β and the remaining error ε.

Here, GLMs are used to determine whether the toxic pressure of mixtures significantly contributes to the value of the selected biological index. The significance of the mixture pressure will be evaluated compared to other water quality variables such as nutrients.
To evaluate the significance of predictor variables, stepwise model selection was used. Two methods were combined by the algorithm (stepAIC from the R-package MASS): forward and backward selection. Akaike Information Criterion (AIC), a parameter for the likelihood of the model, was used to select the optimal model. In forward model selection, the algorithm starts with the most simple model (no predictor variables) and evaluates which predictor variables in the model lower the AIC. Backwards model selection starts from the most complex model (all predictor variables and their interactions) and evaluates the reduction in AIC by removing predictor variables. The algorithm proceeds step by step, adding/removing the predictor variable with the most impact on the AIC. The model with the lowest AIC is considered the optimal model i.e. the most likely model.

Statistical models are simplifications of reality, based on a number of assumptions. To be certain of the validity of statistical models, these assumptions need to be validated. For the GLM, the following model assumptions were evaluated:

- Multicollinearity: high correlations between multiple predictor variables is an issue as this distorts the relationships between predictor and response variable. Only one of colinear parameters should be included. To evaluate multicollinearity, the Variance Inflation Factor (VIF) was used. For the monitoring data, a VIF of 5 was used as a boundary for issues with multicollinearity.
- The error structure of the GLM should be correctly distributed e.g. follow a normal distribution. The model residuals should show Homoscedasticity (same error across all variables) and show no pattern after the model has been applied. These model assumptions are visually evaluated using residual versus predictor plots, QQ-plots and observed versus predicted plots.

1.4. MIXTURE RISK

For the cumulative risk assessment, the focus was made on those datasets supporting the identification of mixtures of concern for cumulative risk and/or ecological risk (risk identified) which would merit a review using the approach of the CEFIC MIAT decision tree for assessing effects from exposures to multiple substances, which may include an assessment of Toxic Unit (TU). For the cumulative risk assessment, two databases were selected: i.e. the Eau-France – Adour/Garonne region and the Rhine database. The Eau-France Adour/Garonne region represents a dataset with samples covering a large area and substances from different sources which are covered by different legislations. The Rhine database represents a hot-spot example, with a limited number of sampling points.

1.4.1. CALCULATION OF MIXTURE RISK

As a conservative first tier assessment, cumulative risks of a mixture was assessed with the concentration addition-based Toxic Unit approach. This approach assumes that substances act via a common mode of action. In addition, it is assumed that all substances contribute to the mixture pressure, even at very low concentrations, proportionally to its Toxic Unit. The concentration addition model is generally considered to be a conservative approach (e.g. Cedergreen et al. 2008). The Toxic Unit of a chemical (TU_i) is expressed as the measured environmental concentration of the chemical divided by an effect concentration (e.g. EC_{10}, NOEC, EC_{50}) of that chemical (e.g. TU_i=concentration/EC_{10}). Cumulative pressure imposed by a mixture is then expressed by summing the individual substance TU_i to the mixture toxic unit ($TU_{mix}=\Sigma TU_i$). Although the TU method has originally been developed for summing TU at the species level (i.e. using effect levels), in the context of environmental regulation, the concept is often used to sum Toxic Units based on environmental threshold levels such as PNECs or HC_5s (i.e. sum concentration/ PNEC or HC_5). For the current analysis, it was decided to use the HC5 threshold instead of PNECs. This is because PNECs were deemed less appropriate to understand mixture impacts because PNECs are not predictive of effects as they imply a regulatory based uncertainty assessment in addition to the effect assessment.

In the present study, the Hazard Quotient (HQ)/Hazard Index (HI) approach has been used to estimate cumulative risks in the current study (Price et al. 2012, OECD 2018). The Hazard Quotient (HQ) is the ratio of an environmental concentration relative to the environmental threshold for an individual substance (eq. 1). The environmental threshold level selected in the present study is the HC5s. The Hazard Index (HI) is the summation of the HQ of all substances in the mixture (eq. 2).

$$HQ_i = \frac{c_i}{HC5_i} \quad \text{(Eq. 1).}$$

$$HI = \sum HQ_i \quad \text{(Eq. 2)}$$

Within the HQ/HI approach, a HI>1 indicates that there are unacceptable risks associated with the cumulative exposure.

1.4.2. SELECTION OF EFFECT DATA

In practice, HC_5 values were calculated based on log chronic NOEC/EC_{10} and the slope of the species sensitivity distribution (SSD) collated by Posthuma et al. (2019). The dataset of Posthuma et al. (2019) consists of parameters (median and standard deviation of log-transformed toxicity data) of log-normal SSDs for 12 386 chemicals. Separate parameters are published for both the acute and chronic SSD. Toxicity data for the derivation of the SSDs was collated from different sources, such as the US EPA's ECOTOX database, REACH data. For substances for which insufficient toxicity data could be extracted, Posthuma et al. (2019) used read-across approaches to estimate the remaining SSD-parameters. SSDs parameters were derived when toxicity data for at least 3 species was retrieved. Remaining datagaps between acute and chronic SSDs were tackled with extrapolation factors (Posthuma et al. 2019). We extracted the necessary data from the Posthuma et al. (2019)-database based on CAS-numbers. If an exact match was not found, the search was repeated based on the substance name. For a few substances, no match based on CAS-number of substance name could be found, for these substances an environmental threshold level was derived from the WFD-directive (i.e. annual average-environmental quality standard (AA-EQS), EC 2013/39/EU).

Table 3 shows the different quality categories for the chronic SSDs selected from Posthuma et al. (2019). For the Rhine dataset, 67% of the chronic SSDs were not extrapolated (i.e. they were derived based on chronic toxicity data). 29% of the chronic SSDs were extrapolated based on acute EC_{50} SSD and 4% based on the acute NOEC SSD. For the Adour-Garonne dataset, 51% of the chronic SSDs were not extrapolated (i.e. they were derived based on chronic toxicity data). 29% of the chronic SSDs were extrapolated based on acute EC_{50} SSD and 7% based on the acute NOEC SSD. For 10% of the substances, the chronic SSD was extrapolated from a poorly presented Acute SSD, implicating that the acute SSD was derived from read across or that the acute SSD contained only 1 toxicity data point. For the remaining substances, there was no match found within the Posthuma database, for these data other sources were used to obtain an effect threshold.

Table 3: Data quality of SSD parameters extracted from the database of Posthuma et al. (2019) for the Adour Garonne and Rhine dataset. Numbers indicate number of substances belonging to each category.

SSD extrapolation category	Number of species in SSD	Rhine	Adour-Garonne
Chronic NOEC not extrapolated	Officially enough species (>10) for ERA with SSDs[a]	34	36
	Enough species (6-10) for ERA with SSDs	-	1
	Marginally enough species (3-5) for ERA with SSDs	-	2
Chronic NOEC extrapolated from Acute EC50[b]	Officially enough species (>10) for ERA with SSDs	12	11
	Enough species (6-10) for ERA with SSDs	3	4
	Marginally enough species (3-5) for ERA with SSDs	-	7
Chronic NOEC extrapolated from Acute NOEC[c]	Officially enough species (>10) for ERA with SSDs	2	2
	Marginally enough species (3-5) for ERA with SSDs	-	3
Chronic NOEC extrapolated from poorly presented Acute SSD[d]	Acute EC50 for 1 species	-	1
	Read across	-	7
Other sources: WFD-EQS, Restriction dossier		-	3
Total number of substances considered in cumulative risk estimation		**51**	**76**

[a] 'Officially enough species' refers to the minimal sample size put forward for the use of statistical derivation methods as described in the Technical Guidance Document on Risk Assessment (EC 2003).
[b] An extrapolation factor of 10 was applied by Posthuma et al. (2019) to extrapolate from acute EC50 to chronic NOEC, i.e. the acute EC50s were divided by 10.
[C] An extrapolation factor of 9 was applied by Posthuma et al. (2019) to extrapolate from acute NOEC to chronic NOEC, i.e. the acute NOECs were divided by 9.
[d] An extrapolation factor of 10 was applied by Posthuma et al. (2017) on the median acute EC50 calculated from read-across or the poorly represented acute SSD, and a slope of 0.7 was assumed (Posthuma et al. 2019).

Table 4 and Table 5 give an overview of the 5% hazardous concentrations calculated from the SSD-parameters published by Posthuma et al. (2019).

Table 4: Overview of 5% hazardous concentratiog (HC5) calculated based on the SSD parameters published by Posthuma et al. (2019) for the substance detected in the Rhine database, ranked from lowest HC5 to highest HC5.

Toxicity range	Chemical (toxicity threshold in µg/L)
1 – 10 ng/L	benzo(ghi)perylene (3.3 ^{e}-3); ethyl-parathion (4.1 ^{e}-3); diazinon (7.8 ^{e}-3)
11-100 ng/L	azinphos-ethyl (1.1 ^{e}-2); benzo(b)fluoranthene (5.2 ^{e}-2); benzo(k)fluoranthene (5.2 ^{e}-2); indeno(1,2,3-cd)pyrene (8.3 ^{e}-2)
101-1000 ng/L	malathion (1.9 ^{e}-1); Isoproturon (2.3 ^{e}-1); benzo(a)pyrene (2.3 ^{e}-1); chlorfenvinphos (2.9 ^{e}-1); trifluralin (3.7 ^{e}-1); diuron (3.9 ^{e}-1); methyl-parathion (4.4 ^{e}-1); Methabenzthiazuron (768); Linuron (930)
1-10 µg/L	cadmium dissolved (1.0); Metolachlor (1.1); Fenitrothion (1.9); Hexachlorobutadiene (2.0); Fluoranthene (2.1); Simazine (2.1); Atrazine (2.2); atrazine-desethyl (2.2); terbuthylazine (2.5); nickel (Ni) dissolved (2.8); copper (Cu) dissolved (3.9); zinc (Zn) dissolved (6.2); Monolinuron (6.8)
11-100 µg/L	chromium (Cr) total (11.9); 1,2,3-trichlorobenzene (12.9); Bentazone (13.3); Alachlor (15.5); 1,2,4-trichlorobenzene (16.3); lead (Pb) dissolved (18.4); Dimethoate (20.0); Chlorotoluron (21.3); 1,3,5-trichlorobenzene (29.4); 1,2-dichlorobenzene (33.1); Dichlorprop (45.0); 1,4-dichlorobenzene (57.2); 2,4-dichlorophenoxy acetic acid (86.5)
101-1000 µg/L	Naphthalene (104); MCPA (315); Mecoprop (599); Benzene (711); Trichlorethene (756)
>1000 µg/L	Tetrachloromethane (2547); Trichloromethane (3194); 1,2-dichloroethane (3492) ;MTBE (methyl tert-butyl ether) (5207)

Table 5: Overview of 5% hazardous concentratiog (HC5) calculated based on the SSD parameters published by Posthuma et al. (2019) for the substance detected in the Adour-Garonne database, ranked from lowest HC5 to highest HC5.

Toxicity range	Chemical (toxicity threshold in µg/L)
<1 ng/L	Tributylstannane (6.3^{e}-4)
1 – 10 ng/L	-
11-100 ng/L	N-Cyclopropyl-N'-(1,1-dimethylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine (1.1^{e}-2); estrone (1.2^{e}-2); Total DDDpp', DDEpp', DDTop', DDTpp' (2.5^{e}-2)[a]
101-1000 ng/L	Anthracene (1.3^{e}-1); PBDE (1.4 ^{e}-1); Ibuprofen (2.3 ^{e}-1); Benzo(a)pyreen (2.3 ^{e}-1); Lindane (3.7 ^{e}-1); 2-Hydroxy Ibuprofen | Hydroxyibuprofen (7.1 ^{e}-1); Triclocarban (7.9 ^{e}-1)
1-10 µg/L	Perfluoroctaansulfonaat (PFOS) (1.1); Triclosan (1.1); Chloroacetic acid (1.3); Sulfametoxazol (1.7); paracetamol (1.9); 4-Nonylphenol, Branched (2.0); Hexachlorobutadiene (2.0); Fluoranthene (2.1); Karbamazepin (2.1); 4-(1,1,3,3-Tetramethylbutyl)phenol (2.3); Cyanide (3.2), Hexachlorobenzene (3.3); (1 alpha,2 alpha,3 beta,4 alpha,5 beta,6 beta)1,2,3,4,5,6-Hexachlorocyclohexane (3.5); Pentachlorphenol (3.5); Bisphenol-A (7.4); 1,2,3,4,5-Pentachlorobenzene (7.4); benzyl butyl phthalate (9.5)
11-100 µg/L	1,2,3-Trichlorobenzene (12.9); 1,2,4-Trichlorobenzene (16.3); bis(2-ethylhexyl) phthalate (16.7); dibutyl phthalate (17.3); Niflumic acid (17.3); 4-Chlorophenol (20.1); Oxazepam (25.0); 1,3,5-Trichlorobenzene (29.4); Bromomethane (35.1); Norethindrone (41.6); 2,4-Dinitrotoluene (48.9); Fenofibrate (51.6); Ketoprofen (60.1); Trichlorobenzene (65.5); 1,1'-Oxybis[2,4-dibromobenzene] (73.9); PFHxA (perflurohexanoic acid) (86.0)[b]; PFHxS (perfluorohexane-1-sulphonic acid) (86.0)[b]; 10,11-Dihydro-10,11-epoxycarbamazepin (93.0); p-cresol (99.7)
101-1000 µg/L	Naphthalene (104); o-cresol (105); 2,6-Dinitrotoluene (128); 1,2-Dibromoethane (134); (Chloromethyl)oxirane (159); 1,1,2,2-tetrachloroethane (275); sodium [2-[(2,6-dichlorophenyl)amino]phenyl]acetate (305); Nitrobenzene (333); Tetrachloroethylene (514); propyl 4-hydroxybenzoate (515); Diethyl phthalate (636); ethyl 4-hydroxybenzoate (671); Carboxyibuprofen (674); Cyclophosphamid (685); Pentadecafluorooctanoic acid (695); Benzene (711); trichloroethylene (756); dimethyl phthalate (782)
>1000 µg/L	1,1,2-Trichloroethane (1184); Tetrachloromethane (2547); Trichloromethane (3194); 1,2-Dichloroethane (3492); tert-butyl methyl ether (5207); Vinyl chloride (7433); Perchlorate (13791); Dichloromethane (14854)

[a] For "Total DDDpp', DDEpp', DDTop', DDTpp'", the AA-EQS was selected (EC 2013/39/EU).

[b] For PFHxA and PFHxS, the lowest acute EC50 (i.e. EC50 of 86 mg/L for *Scenedesmus subcapitatus*) was selected from the Annex XV restriction report[2] on which an assessment factor of 1000 was applied.

[2] https://echa.europa.eu/documents/10162/c4e04484-c989-733d-33ed-0f023e2a200e)

The current approach with a first level assessment using the Hazard Index approach based on summing of TU of HC_5s builds in **several levels of conservatism** relative to:

1) The **use of HC_5s from Posthuma et al. (2019)** instead of PNECs: due to the incorporation of an assessment factor, PNECs tend to be lower than the HC_5s used in the present study. Hence, when using PNECs, risks will be 'flagged' at lower concentrations. However, this depends on the (group of) chemicals: **for some of the driving chemicals**, the HC_5 derived from the dataset of Posthuma et al. (2019) appeared to be **very conservative relative to other sources**. For instance, for the polycyclic aromatic hydrocarbon benzo(ghi)perylene, the HC_5 derived from Posthuma et al. (2019) was observed to be 100-fold lower than the PNEC value derived based on the target lipid model (Redman et al. 2017). Refining the Hazard Index/Hazard Quotient approach using target lipid model based PNECs for PAH, while using the HC_5s derived from Posthuma et al. (2019) for all other substances, resulted in substantially lower risk predictions. While 54% of the mixture exposures were predicted at risk in the initial analysis for the Rhine dataset, after the refinement of the effect levels for PAHs, only 16% of the mixture exposures were predicted to be at risk. Further refinements on the key contributors (e.g. by considering toxic mode of action) is likely to further reduce the number of mixtures at (potential) risk. This example clearly shows that the dataset of Posthuma et al. (2019) can be used as a first tier assessment, but that further refinements in the methodology can shed a different light on actual risks.

2) **Bioavailability of inorganics and organics**: the bioavailability and toxicity of both inorganics and organics can be dependent on the characteristics of the receiving water. For instance, the toxicity of ionizable organic compounds (IOCs) is highly dependent on pH, mainly because the uptake and bioaccumulation of IOCs is pH dependent (Escher et al. 2020). Another example are metals, for which toxicity is highly dependent on physico-chemistry characteristics such as Dissolved Organic Carbon, pH, and hardness (Mebane et al. 2020). Using generic environmental threshold levels for mixture pressure assessments leads inevitably to a certain degree of conservatism relative to site-specific environmental threshold levels. These site-specific environmental threshold levels may for instance be calculated with ion-trapping and toxicokinetic models (IOCs) or biotic ligand models (metals). However, site-specific bioavailability corrections are often 'data-hungry' and complex requiring expert-level knowledge, although simplified models are available for certain compounds.

3) Additional conservatism is incorporated in the assessment by applying the **concentration addition method on an environmental threshold level** such as the HC_5. It has been shown that this method incorporates a substantial margin of safety relative to more complex methods such as applying the concentration addition method at the species or trophic level or relative to the independent action model (Backhaus & Faust 2012, Gregorio et al. 2013, Van Regenmortel et al. 2017, Nys et al. 2017).

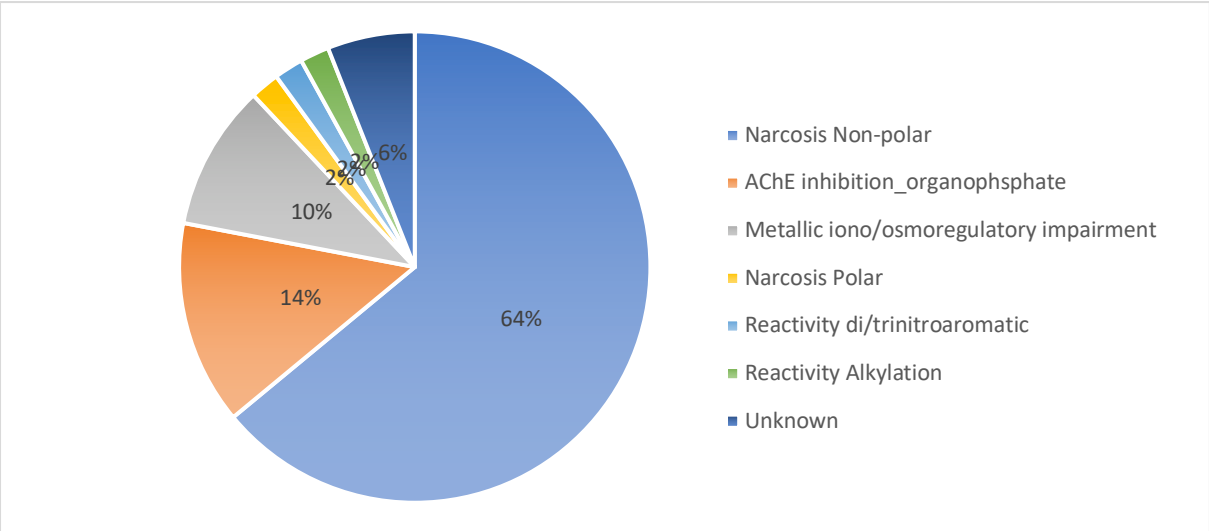


Figure 12: The different mode of actions in the mixtures observed in the Rhine basin demonstrates that a toxic unit approach is a conservative lower tier assessment as in reality, additivity is only to be assumed for substances in mixtures with the same mode of action. Percentage indicates the percentage of substances with a specific mode of action.

1.4.3. DEALING WITH NATURALLY OCCURING SUBSTANCES

Naturally occurring substances, such as metals, may even in pristine situations result in a geochemical or biological background concentration. It has been argued that some of the risk evaluation methods, such as the HI/HQ approach, are not suitable for naturally occurring substances, as they might predict risks below natural background concentrations (Van Regenmortel et al. 2017, Nys et al. 2018). This is further complicated in the case of essential elements for which regulatory limits might be set in the range where deficiency occurs (Meyer et al. 2015). For instance, Nys et al. (2018) showed that 13% of water samples from a database representing geochemical baseline concentrations in pristine European waters (i.e. the FOREGS database) are considered to be at risk using the HI/HQ approach (i.e. HI>1), even after correction for local bioavailability conditions (using BLM) when only the following 5 metals are considered: Zn, Ni, Cu, Cd and Pb. This observation of potential concerns predicted with the HI/HQ approach at metal background concentrations is further corroborated by the HQ calculations using the lower 10^{th} percentile of metal concentration in the geochemical baseline FOREGS database relative to the HC_5 derived from the Posthuma et al. (2019) database. These lower 10^{th} percentiles of metal concentration in the geochemical baseline FOREGS database are considered to be background concentrations of metals in European waters (Table 6). Especially for Cu, Zn and Ni relatively high HQ values are observed at background concentrations, with HQs ranging between 0.22 (Cu) and 0.67 (Ni) (Figure 13). The HI associated with the background concentrations in European waters is 1.4, indicating that even at background concentrations the HI/HQ approach calculates unacceptable risks of mixture exposure.

To account for background concentrations, the added concentration approach has been used for naturally occurring substances. The added (risk) approach assumes that species are fully adapted to the natural background concentration and therefore contributes to the toxicity. This approach accounts for background concentrations of naturally occurring substances by subtracting the natural background from the measured environmental concentration.

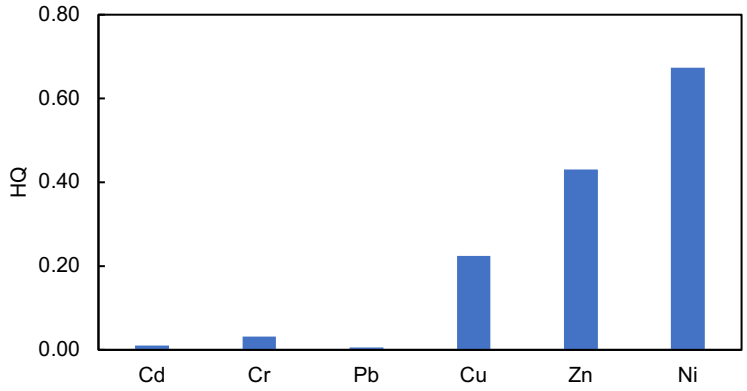


Figure 13: Predicted risk associated with background concentrations of metals. Risks are expressed as Hazard Quotient (HQ=conc/HC5). Metal background concentrations are the 10th percentile of metal concentrations derived from the FOREGS database (Table 6). The FOREGS database represents geochemical baseline conditions in pristine waters across Europe. The HC5 used to calculate the HQ was derived from Posthuma et al. (2019).

Table 6: Overview of background concentrations[a] of metals in European surface waters.

Metal	Background concentration (µg/L)
Cd	0.01
Cr	0.38
Pb	0.09
Cu	0.88
Zn	2.68
Ni	1.91

[a] Background concentrations are represented here as the lower 10th percentile of metal concentrations in the FOREGS database

1.4.4. CEFIC MIAT DECISION TREE

The HQ/HI approach has also been integrated in the CEFIC-MIAT decision tree (Price et al. 2012). However, the environmental threshold level for the Tier 1 assessment proposed by Price et al. (2012) is the PNEC or EQS, instead of the HC_5. In analogy with the CEFIC-MIAT decision tree, a differentiation was made between risks driven by individual substances and mixtures based on the concept of Maximum Cumulative Ratio (MCR). The MCR is the ratio of the total toxicity to the largest toxicity from a single chemical stressor (Eq. 3, Price & Han 2011).

$$MCR = \frac{HI}{\max HQ_i} \qquad \text{(Eq. 3)}$$

The MCR can range from 1 to *n* (with *n* the number of chemicals in the exposure). Values close to 1 indicate that one chemical dominates the mixture toxic pressure. An MCR equal to *n* indicates that the receptor is exposed to equitoxic doses of all chemicals.

The grouping of mixture exposures into different categories based on the HI and MCR helps to identify the mixtures of concern and support risk management decisions (Price et al. 2012). In practice, four exposure groups are distinguished.

- Group I: Combined exposures that are a potential concern because one or more individual chemicals are a concern: i.e. at least one of the $HQ_i>1$
- Group II: Combined exposures where there is a low concern for both individual chemicals and for their combined effects: i.e. HI<1
- Group IIIa: combined exposures where there is a low concern for individual chemicals but there is a potential concern for the combined effects; one chemical provides the majority of toxicity of the combined exposures: i.e. all $HQ_i<1$, HI>1 and MCR<2
- Group IIIb: combined exposures where there is a low concern for individual chemicals but there is a potential concern for the combined effects; no one chemical is dominating, i.e. all $HQ_i<1$, HI>1 and MCR>2

Figure 14 shows the different groups as a function of the hazard index and maximum cumulative ratio. In the context of environmental regulation, Group I exposures are regulated by single-substance regulations. For Group II mixtures, risks are considered to be of 'low concern'. Group III exposure are of main concern in the context of mixture toxicity. This is because the cumulative risk observed in these exposures cannot be assessed by current substance-by-substance legislation. Group IIIa mixtures can be remediated by targeting the substance that is responsible for the majority of the cumulative risk. Group IIIb represent the complex mixtures, which should be further addressed.

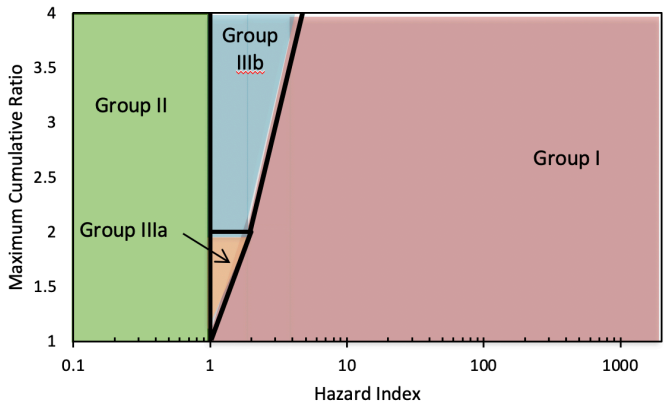


Figure 14: Plot showing the Maximum Cumulative Ratio as a function of the Hazard Index. The colored regions indicate the different exposure groups to differentiate between mixture exposure risks (see text for interpretation of different groups).

1.4.5. REFINEMENT BASED ON MODE OF ACTION

The Hazard Index/Hazard Quotient approach is a rather conservative approach to estimate cumulative risk of mixtures (Backhaus & Faust 2012, OECD 2018, EFSA 2019). It can be expected that the underlying assumption of the concentration addition mixture reference model that all substances in the mixtures have a common mode of action is often violated for complex environmental mixtures. The competing concept of independent action (IA), maybe used in higher tier assessments, when there is an indication of multiple modes of action (e.g. Backhaus

& Faust 2012). When applied on SSDs of the individual mixture constituents, the IA calculates the mixture pressure expressed as multi-substance potentially affected fraction based on the product of the fraction of species not affected by each of the individual mixture constituents (Traas et al. 2002). A hybrid method ($msPAF_{MoA}$) combines the hazard index approach for substances with the same mode of action with the independent action model to calculate an msPAF for complex mixtures with both shared and different modes of actions (Traas et al. 2002; De Zwart and Posthuma 2005, Parkerton et al. 2018), such as in the environmental samples under study. Mode of Actions of the different substances were extracted from the database of Kienzler et al. (2017). Preference was given to the mode of action from the EPA Mode of Action and Toxicity database (MOA_{tox}). If no Mode of Action from MOA_{tox} was reported, the MoA of the ASsessment Tool for Evaluating Risk (ASTER) QSAR application was selected.

In practice, a refinement of the mixture pressure calculations was done for all Group III mixtures using the hybrid $msPAF_{MoA}$ which combines calculation of a HI per MoA (concentration addition based) with the independent action model to calculate a msPAF for the entire mixture exposure. This is done in three steps. First, a separate Hazard Index for each of the modes of action *j* (HI_{MoAi}) was calculated, by summing all Hazard Quotients of a specific Mode of Action (MoA_j). The HI_{MoAj} was calculated using the median chronic NOECs, extracted from Posthuma et al. (2019) as denominator ($HI_{Median\ NOEC,MoAi}$j; Eq. 4).

$$HI_{median\ NOEC,MoA_j} = \sum HQ_{medianNOEC,MoA_j} = \sum \frac{c_{i_{MoAj}}}{median\ chronic\ NOEC_{i_{MoAj}}} \qquad \text{(Eq. 4)}$$

Second, the Potentially Affected Fraction of the species by MoA *j* (PAF_{MoAj}, was then calculated assuming a log-normal distribution, with the excel function NORMDIST using the log-transformed $HI_{median\ NOEC,MoAj}$. When a Mode of Action was represented by several substances the slope for PAF_{MoAj} calculation was calculated by averaging the SSD slopes of all substances belonging to the specific MoA. If only one substance was representing a certain Mode of Action, the substance-specific SSD slope, extracted from Posthuma et al. (2019) was used. Finally, the multi substance Potentially Affected Fraction ($msPAF_{MoA}$) of the mixture exposure was calculated by multiplying the fraction of species not affected by each of the Modes of Action present in the mixture exposure (eq. 5)

$$msPAF_{MoA} = 1 - \prod(1 - PAF_{MoA_i}) \qquad \text{(Eq. 5)}$$

The hybrid method can be seen as a higher tier assessment compared to the HI/HQ approach applied as conservative first tier, because the concentration addition method tends to be more conservative and less accurate compared to the independent action model for mixture exposures with different modes of actions both at the level of mixture effects as on mixture risks (e.g. Faust et al. 2013, Van Regenmortel et al. 2017, Nys et al. 2018).
It should be noted that similar as for the concentration addition model in the HI/HQ approach, the application of the independent action model on the SSD to calculate a msPAF is a pragmatic choice, that is not entirely consistent with theoretical mixture reference. This is because both mixture reference models are theoretically consistent when applied on the species level (i.e. used for a specific species instead of an SSD). However, applying the mixture reference models in a theoretical consistent way for complex mixtures is not practical as suggested in higher tiers for example by OECD (20180, as these approaches are very data-demanding and computationally intensive (at least for the independent action model) (e.g. Van Regenmortel et al. 2017).

2. TECHNICAL ANNEX B: ENVIRONMENTAL CO-EXPOSURE

2.1. WATERBASE

2.1.1. DATA PROCESSING

The original database is available as a long list in the format i.e. one individual measurement for one chemical per row. A first step was to aggregate this to 'samples' i.e. chemicals that co-occur as part of the same mixture.

- Chemicals that were measured on the same location and in the same month and year were considered part of the same sample.
- If multiple measurements of the same chemical were available per sample, the maximum of the values was taken.
- Measurements below the detection limit were set to half the reported detection limit.

This resulted in a dataset of 579,882 rows (samples) and 452 columns (chemicals). The dataset contains measurements from 1974 to 2019, but the most measurements are available from 2000 onwards. Data from 36 European countries are present, **most notably from France** (176,534 samples), UK (76,373), Italy (55,724), Poland (36,575), Finland (36,503), Austria (36,955) and Hungary (22,635).

This dataset consisted of incomplete samples i.e. not all 452 chemicals were present in the samples. For the PCA analysis, a dataset with complete samples is required. Further data processing was done and a final dataset for analysis was prepared with 27,667 samples (i.e., mixtures) and 72 chemicals. The samples in this dataset were taken between 2008 and 2018 in France only. The final dataset contains 58 agrochemicals, 10 industrial chemicals, 4 PAHs and 3 metals (some chemicals have dual use e.g. agrochemical and industrial). 27 of the 72 chemicals are priority pollutants. Before PCA analysis, the chemical concentrations were log-transformed and standardized.

2.1.2. OBSERVED MIXTURES

The PCA analysis explained a large part of the variance in the dataset: 68.7% of the variance was explained by the first three components. The biplot indicated a satisfactory PCA analysis, with clear clusters of sites (points in the biplot) and the chemicals showing clear correlations with the principal components (Figure 15).

The Varimax loadings give a quantitative indication for which chemicals are most correlated with the principal components (Table 7). The first component is a mix of chemicals belonging to the different chemical groups in the dataset (agrochemicals, industrial chemicals, metals and PAHs). The positive varimax scores indicate a mixture of a broad group of chemicals with no immediate explanation for their co-occurrence (but see next section). On the other side of the first component, a small subset of chemicals can be identified: chlorsulfuron, simazine, metribuzin, heptachlor, dichloromethane. Possibly, this reflects a more agricultural mixture, although dichloromethane is an industrial chemical. Nevertheless, the analysis indicates that chemicals on the positive side of the first principal component tend to not co-occur with chemicals on the negative side. The second principal component is similarly a mixture of all chemical groups, although metals are less prominent. Again, the positive varimax scores

indicate a wide mixture of co-occurring chemicals while the negative side suggests a smaller mixture (all agrochemicals: chlorpyrifos, terbutryn, DDT, alachlor) that does not co-occur with the others. The third principal component contains almost exclusively agrochemicals, with no chemicals with a negative varimax scores. This suggests the PPP mixture only occurs in areas with high agriculture pressure.

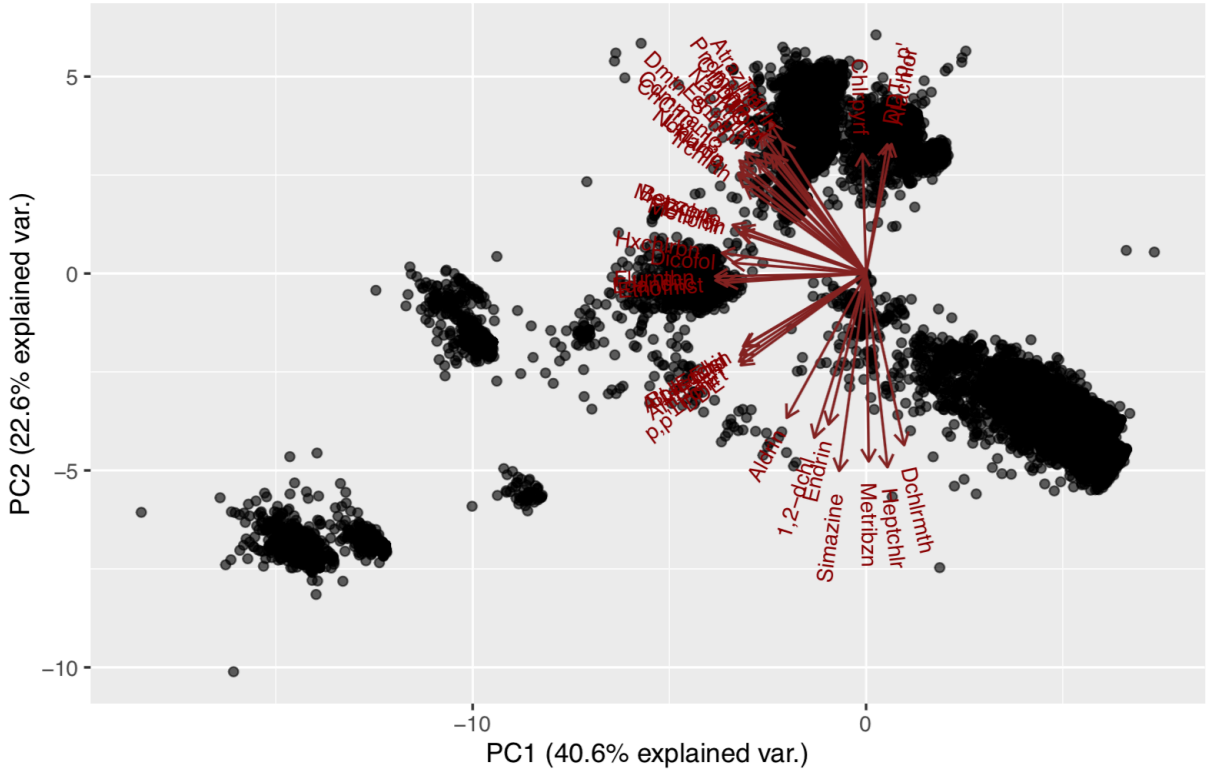


Figure 15: Biplot for the Waterbase PCA.

Table 7: Varimax loading scores for the three principal components of the Waterbase PCA. Only the varimax scores >0.4 (absolute value) are shown.

Name	RC1	Group
Cadmium and its compounds	0.96	Metals
Demeton-S-methyl	0.96	PPP
Carbon tetrachloride	0.95	industrial
Pendimethalin	0.95	PPP
Chloroalkanes C10-13	0.94	industrial
Atrazine	0.94	PPP
Linuron	0.93	PPP
Nickel and its compounds	0.92	Metals
Fenitrothion	0.88	PPP
Naphthalene	0.86	PAH
Trichloroethylene	0.84	industrial
Terbuthylazine	0.83	PPP
MCPA	0.83	PPP
Trifluralin	0.82	PPP
DDT, o,p'	0.81	PPP
p,p'-DDD	0.79	PPP
Metazachlor	0.79	PPP
Desethylatrazine	0.76	PPP
Hexachlorobenzene	0.75	PPP, industrial
Fenpropimorph	0.75	PPP
Benzene	0.74	PAH
Diuron	0.73	PPP
Metolachlor	0.73	PPP
Fluoranthene	0.71	PAH
Lead and its compounds	0.69	Metals
Dicofol	0.66	PPP
Ethofumesate	0.64	PPP
Desisopropylatrazine	0.57	PPP
o,p'-DDD	0.45	PPP
Bromacil	0.43	PPP
Pentachlorophenol	0.42	PPP
Chlorpyrifos	0.39	PPP
Chlorsulfuron	-0.38	PPP
Simazine	-0.51	PPP
Metribuzin	-0.62	PPP
Heptachlor	-0.73	PPP
Dichloromethane	-0.74	industrial

Name	RC2	Group
Anthracene	0.89	PAH
p,p'-DDE	0.89	PPP
Aldrin	0.89	PPP
Tetrachloroethylene	0.88	industrial
Isodrin	0.86	PPP
Beta-Endosulfan	0.86	PPP
Alpha-Endosulfan	0.86	PPP
Metamitron	0.81	PPP
Heptachlor epoxide	0.81	PPP
Di(2-ethylhexyl) phthalate (DEHP)	0.81	industrial
Hexazinone	0.8	PPP
Chlorpyrifos-methyl	0.8	PPP
2,4-dichloro-phenoxyacetic acid	0.79	PPP
Simazine	0.76	PPP
Gamma-HCH (Lindane)	0.75	PPP
Alpha-HCH	0.75	PPP
Pentachlorophenol	0.74	PPP
Dichlorvos	0.74	PPP
1,2-dichloroethane	0.74	industrial
Trichloromethane	0.66	PPP, industrial
Lead and its compounds	0.63	Metals
Beta-HCH	0.63	PPP
Fluoranthene	0.62	PAH
Endrin	0.62	PPP
Ethofumesate	0.56	PPP
Metribuzin	0.55	PPP
Heptachlor	0.54	PPP
Hexachlorobenzene	0.51	PPP, industrial
Lenacil	0.51	PPP
Isoproturon	0.51	PPP
Delta-HCH	0.48	PPP
Bromacil	0.47	PPP
Propiconazole	0.46	PPP
Desisopropylatrazine	0.45	PPP
Dieldrin	0.45	PPP
Dicofol	0.4	PPP
Pentachlorobenzene	0.4	PPP, industrial
Desethylterbuthyl-azine	0.4	PPP
Chlorpyrifos	-0.48	PPP
Terbutryn	-0.51	PPP
DDT, p,p'	-0.62	PPP
Alachlor	-0.64	PPP

Name	RC3	Group
Dichlobenil	0.74	PPP
Pentachlorobenzene	0.69	PPP, industrial
Parathion-methyl	0.56	PPP
Dichlorvos	0.5	PPP
Dicofol	0.49	PPP
Bromacil	0.46	PPP
o,p'-DDD	0.45	PPP
2,4-dichlorophenoxyacetic acid, 2-4 D	0.44	PPP
Chlorsulfuron	0.43	PPP
Gamma-HCH (Lindane)	0.42	PPP
Beta-HCH	0.42	PPP
Metribuzin	0.42	PPP

2.1.3. INTERPRETATION

To better understand why certain chemicals co-occur, spatial factors of the sites were taken into account. For each of the sites, information is available on e.g. the region they belong to or the river basin they belong to. Spatial factors are linked to other factors that might explain the observed mixtures e.g. certain regions in France are historically heavy industrialized, subject to intense agriculture or heavily populated, which will determine the chemical mixtures found. To visually explore this, the sites on the PCA biplot are color clustered: sites belonging to e.g. one region are assigned the same color. For the interpretation of the Waterbase PCA, the influence of the Région and the river basin were further explored (Figure 16). When interpreting the clustering, it can be worthwhile to take into account the number of sites per spatial factor. An overview of the number of sites per "Région" and river basins can be found in Table 8.

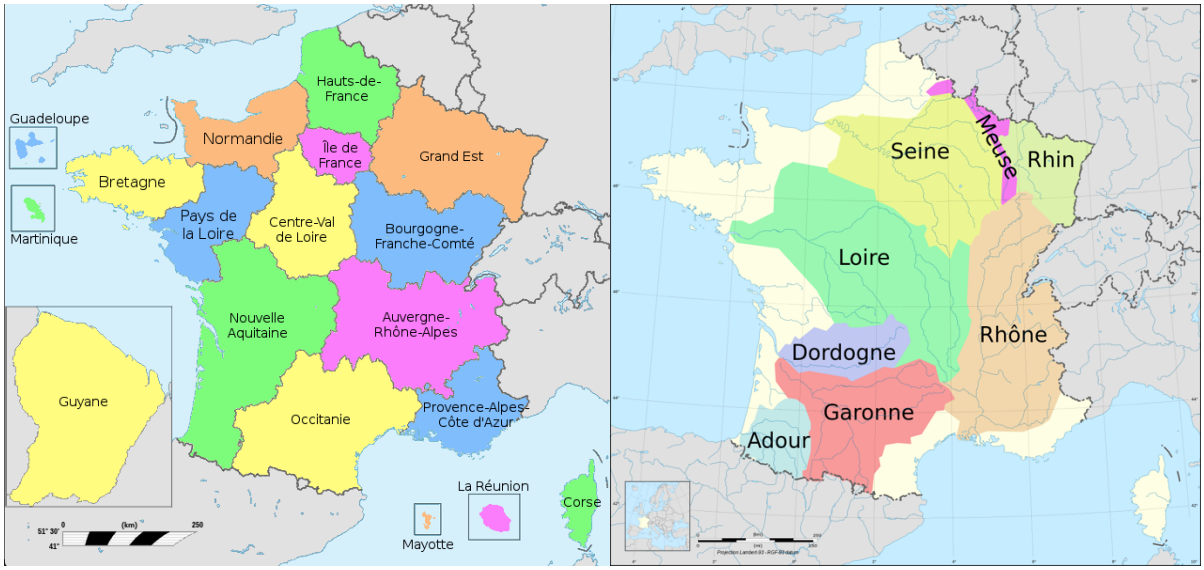


Figure 16: The Régions (left) and river basins (right) in France.

Table 8: Number of sites per Région and river basin in the processed Waterbase dataset.

REGION	SITES
AUVERGNE-RHONE-ALPES	4,821
BOURGOGNE-FRANCHE-COMTE	3,328
BRETAGNE	1,994
CENTRE-VAL-DE-LOIRE	2,233
CORSE	385
GRAND-EST	3,260
HAUTS-DE-FRANCE	933
ILE-DE-FRANCE	991
NORMANDIE	2,251
NOUVELLE-AQUITAINE	1,445
OCCITANIE	1,698
PAYS-DE-LA-LOIRE	1,656
PROVENCE-ALPES-COTE-D'AZUR	2,076

RIVER BASIN	SITES
ESCAUT/SOMME/MER NORD	306
LOIRE & BRITTANY	9,152
MEUSE	533
SAMBRE	61
SEINE & NORMANDY	6,256
RHONE & MEDITERANEAN	9,274
RHIN	1,699
CORSICA	385

Looking at the **Régions** (similar to provinces in France), clear clusters can be identified (Figure 17). These Régions have a clearly distinct history and land use, which can help explain the observed clusters (Figure 16).
The Régions of Ile de France, Hauts-de-France, Normandie, Grand-Est and Centre Val de Loire are all similarly oriented. The area around Paris and the north-east of France has been heavily industrialized for many years (Figure 19). Similarly, agriculture is mainly focused on (the intensive culture) of field crops and to a lesser extent cattle. Mixtures found for these locations have similar profiles, defined by a mixture of agrochemicals, metals and industry metals or by distinct mixtures of agrochemicals.
The Régions of Nouvelle-Aquitaine, Occitanie, Provence-Alpes and Pays de la Loire form another group clearly distinct from the former. Nouvelle-Aquitaine, Occitanie and Provence Alpes have more modern industry and vineyards are a distinct agricultural land use which is nearly absent in the northern parts of France. Pays de le Loire has less vineyards, but this seems to be reflected in the biplot as well as this Région is more distinct from the other three Régions from the same cluster.
Finally, Auvergne-Rhône-Alpes, Bretagne and Bourgogne-Franche-Comté are more in between these two groups. Auvergne-Rhône-Alpes is a distinct group. Around Lyon, heavy industry is present and intensive field cropping is the main agricultural activity. But further away from Lyon, land use becomes more mixed and less intensive. Bretagne is a distinct group because the industry is identified as modern and the agricultural land use is focused on cattle farming. Finally, Bourgogne-Franche-Comté also has both areas with intensive and extensive land use, explaining its intermediate position.

The biplot with clustering for the **river basins** shows similar patterns (Figure 18), which overlap partly with the clustering based on the Régions.
The river basins of the Scheldt, Meuse and Rhin as well as sites near the North Sea cluster together. Here as well, this is explained by the heavy (older) industrial activity in the region and the intensive field cropping practices around these rivers. Sites located in the area around the Seine and in Normandy overlap to a large extent with this cluster, but also contain distinct sites. This was attributed to the sites in Normandy being more focused on livestock production and less heavily industrialized than sites close to the Rhine and Meuse. Similarly, the Rhône only shows a partial overlap, associated with the industry around major city centers (e.g. Lyon) and the mixed industry and agriculture in the rest of the region (mix of field crops, livestock and vineyards). Sites in the Loire river basin and in Brittany are very diverse in the type and intensity of the land use, resulting in a wide range of chemical compositions, as shown by their sites being widely spread in the biplot.

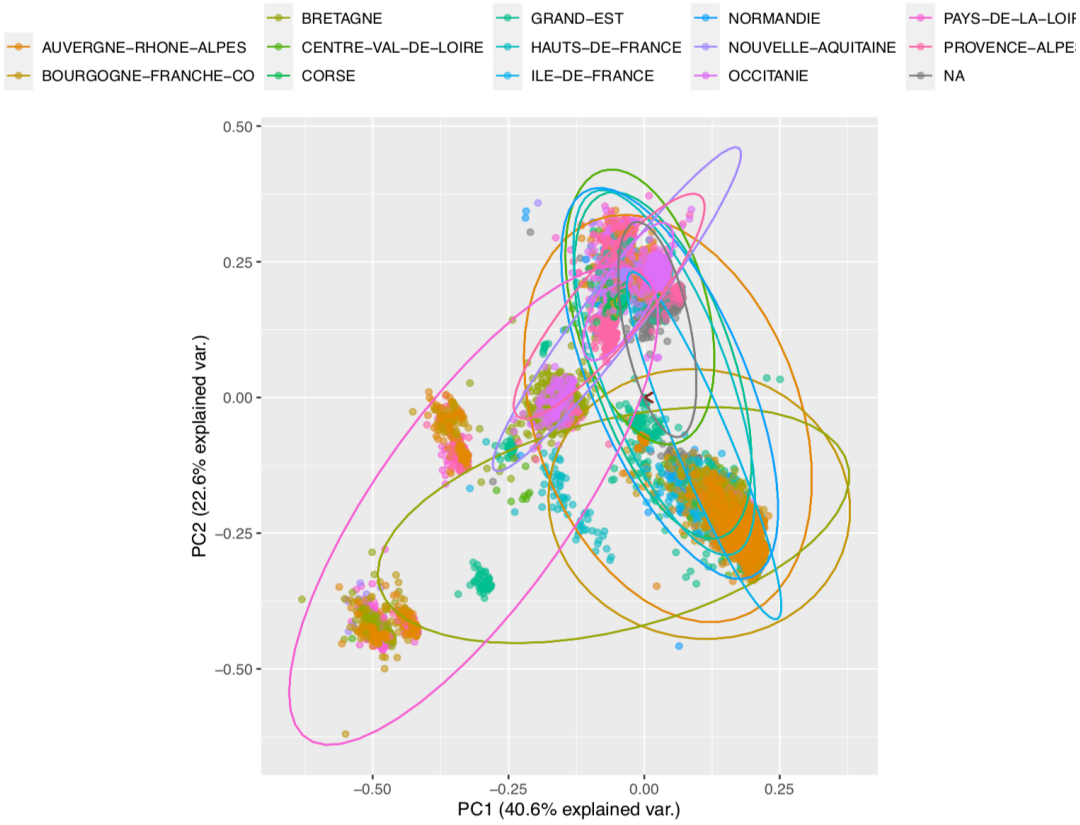


Figure 17: Biplot of the Waterbase PCA, with clustering for the Région the sites are in. Ellipses indicate where 95% of the sites belonging to a specific Région are situated.

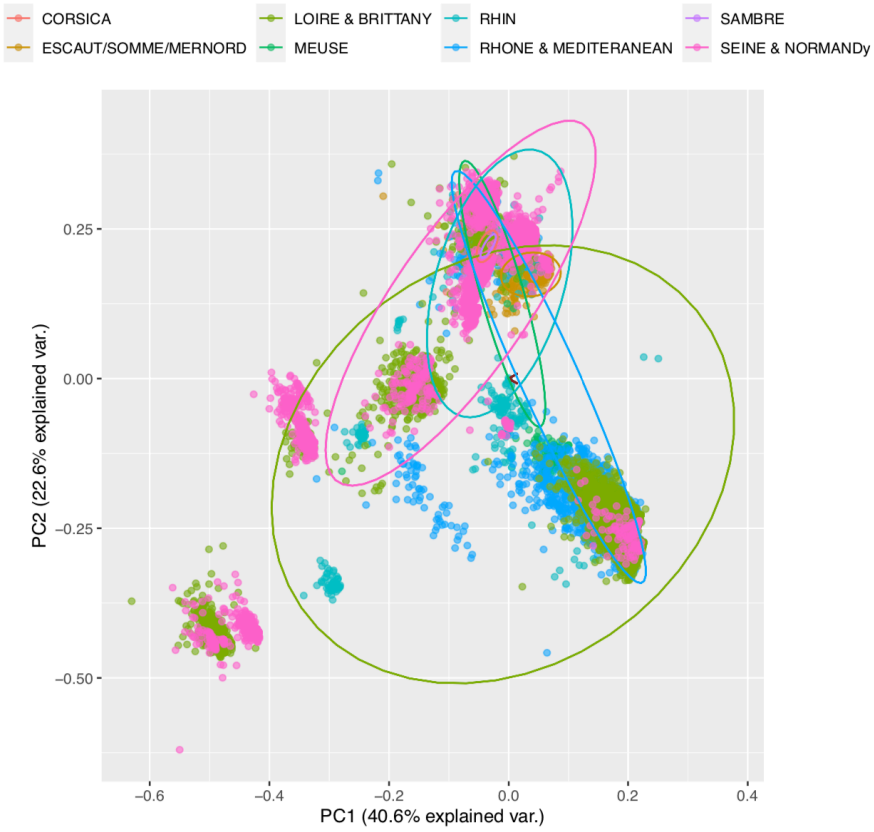


Figure 18: Biplot of the Waterbase PCA, with clustering for the river basin the sites are in. Ellipses indicate where 95% of the sites belonging to a specific river basin are situated.

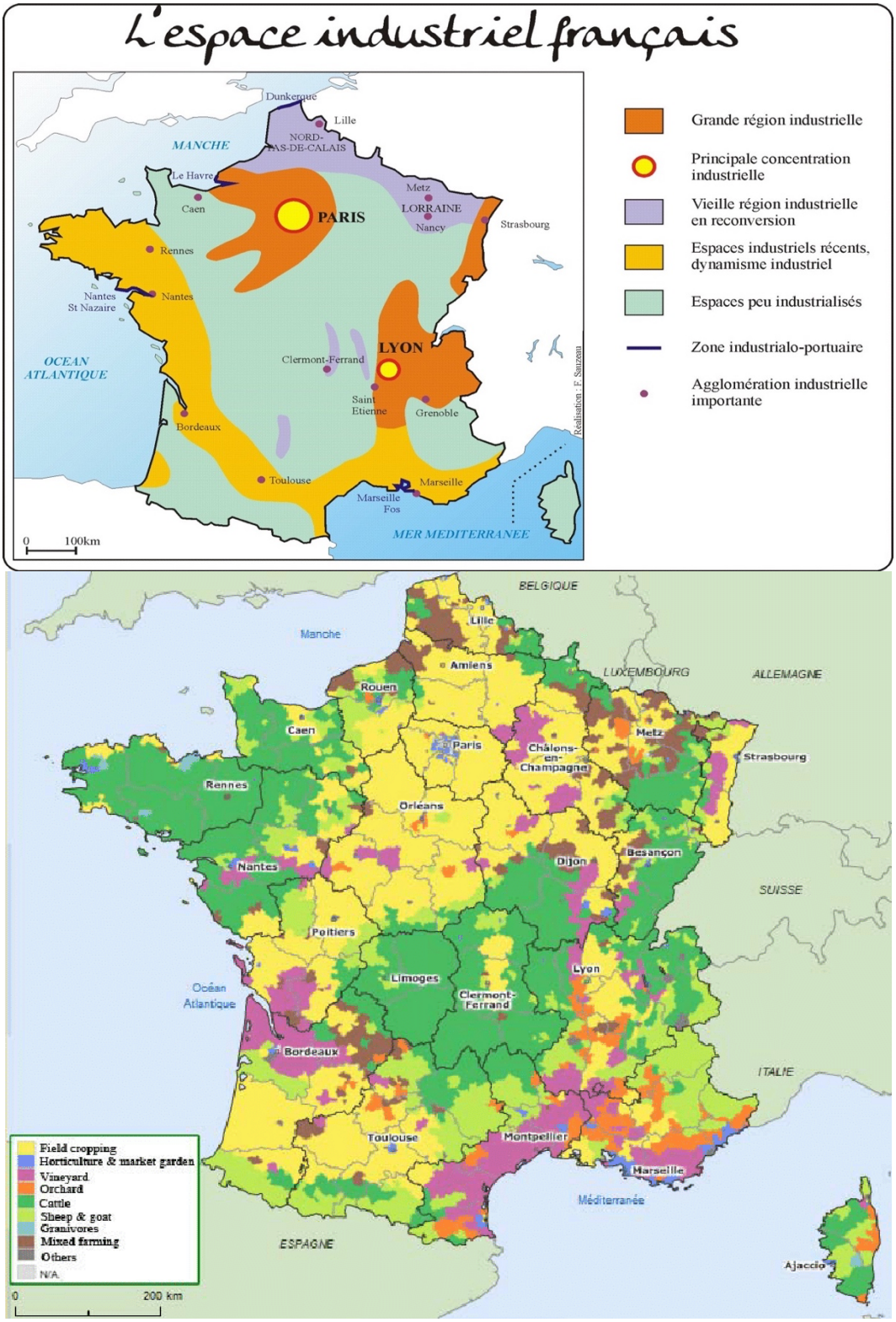


Figure 19: Land use in France: Industry (top) and agriculture (bottom).

Sources: https://www.hgsempai.fr/carto/?p=115; Latruffe et al. 2008.

2.2. RHINE

2.2.1. DATA PROCESSING

The original database is available as a long list in the format of one individual measurement per row. A first step was to aggregate this to 'samples' i.e. chemicals that co-occur as part of the same mixture.

- Chemicals that were measured on the same location and in the same month and year were considered part of the same sample.
- If multiple measurements of the same chemical were available per month and site, the average of the values was taken.
- Measurements below the detection limit were set to the reported detection limit (this has no impact on the PCA).

This resulted in a dataset of 4,182 rows (samples) and 288 columns (chemicals). The dataset contains measurements from 1978 to 2019, but the most measurements are available from 1995 onwards. Data from 16 monitoring locations are present. This dataset consisted of incomplete samples i.e. not all 288 chemicals were present in the samples. For the PCA analysis, a dataset with complete samples is required. Further data processing was done and a final dataset for analysis was prepared with 390 samples and 71 chemicals for 8 monitoring locations. The chemicals in this dataset are mainly priority pollutants. The monitoring locations and chemicals that were not retrieved, did not contain sufficient samples. Before PCA analysis, the chemical concentrations were log-transformed and standardized.

2.2.2. OBSERVED MIXTURES AND INTERPRETATION

The PCA analysis explained a large part of the variance in the dataset: 71% of the variance was explained by the first three components. The biplot indicated a satisfactory PCA analysis, with clear clusters of sites (points in the biplot) and the chemicals showing clear correlations with the principal components (Figure 20).

The Varimax loadings give a quantitative indication for which chemicals are most correlated with the principal components, and indirectly, with each other (Table 17). A varimax threshold of 0.6 was selected to identify the most relevant chemicals. The first component is a mix of chemicals belonging to the different chemical groups in the dataset (agrochemicals, industrial chemicals, metals and PAHs). The positive varimax scores indicate a mixtures of a broad group of mainly industrial chemicals. On the other side of the first component, mixtures or mainly herbicides can be identified. These two groups of mixtures do typically not co-occur. The second principal component are mainly mixtures of plant protection products. The third principal component contains mixtures with agrochemicals and PAH.

Table 9: Varimax loading scores for the three principal components of the Rhine dataset. Only the varimax scores >0.6 (absolute value) are shown

PC1	Substances
0,96	azinphos-ethyl in µg/l
0,96	malathion in µg/l
0,95	chlorfenvinphos in µg/l
0,95	trifluralin in µg/l
0,95	diazinon in µg/l
0,95	dimethoate in µg/l
0,94	alachlor in µg/l
0,94	fenitrothion in µg/l
0,94	ethyl-parathion in µg/l
0,94	methyl-parathion in µg/l
0,94	simazine in µg/l
0,94	1,3,5-trichlorobenzene in µg/l
0,9	terbuthylazine µg/l
0,86	atrazine-desethyl in µg/l
0,84	nickel (Ni) total in µg/l
0,82	copper (Cu) total in µg/l
0,82	zinc (Zn) total in µg/l
0,82	calcium (Ca) in mg/l
0,82	nickel (Ni) dissolved in µg/l
0,8	sulfate (SO4) in mg/l
0,8	naphthalene in µg/l
0,78	sodium (Na) in mg/l
0,78	magnesium (Mg) in mg/l
0,78	dissolved organic carbon (DOC)
0,77	lead (Pb) total in µg/l
0,76	chromium (Cr) total in µg/l
0,76	fluoranthene in µg/l
0,75	total organic carbon (TOC) in mg/l
0,75	copper (Cu) dissolved in µg/l
0,73	cadmium (Cd) total in µg/l
0,71	orthophosphate (o-PO4)
0,68	atrazine in µg/l
0,66	nitrate-nitrogen (NO3-N) in mg/l
0,63	cadmium dissolved in µg/l
0,6	1,2,4-trichlorobenzene in µg/l
0,6	1,2,3-trichlorobenzene in µg/l
-0,59	diuron in µg/l
-0,59	bentazone in µg/l
-0,65	monolinuron in µg/l
-0,65	methabenzthiazuron in µg/l
-0,65	linuron in µg/l
-0,66	benzene in µg/l
-0,75	lead (Pb) dissolved in µg/l
-0,84	trichlorethene in µg/l

PC2	Substances
0,94	mecoprop in µg/l
0,94	dichlorprop in µg/l
0,93	MCPA in µg/l
0,93	2,4-dichlorophenoxy acetic acid
-0,79	1,4-dichlorobenzene in µg/l
-0,79	fenthion in µg/l
-0,72	1,2-dichlorobenzene in µg/l

PC3	Substances
0,69	benzo(ghi)perylene in µg/l
0,66	chlorotoluron in µg/l
0,66	indeno(1,2,3-cd)pyrene in µg/l
0,66	benzo(k)fluoranthene in µg/l
0,63	isoproturon in µg/l

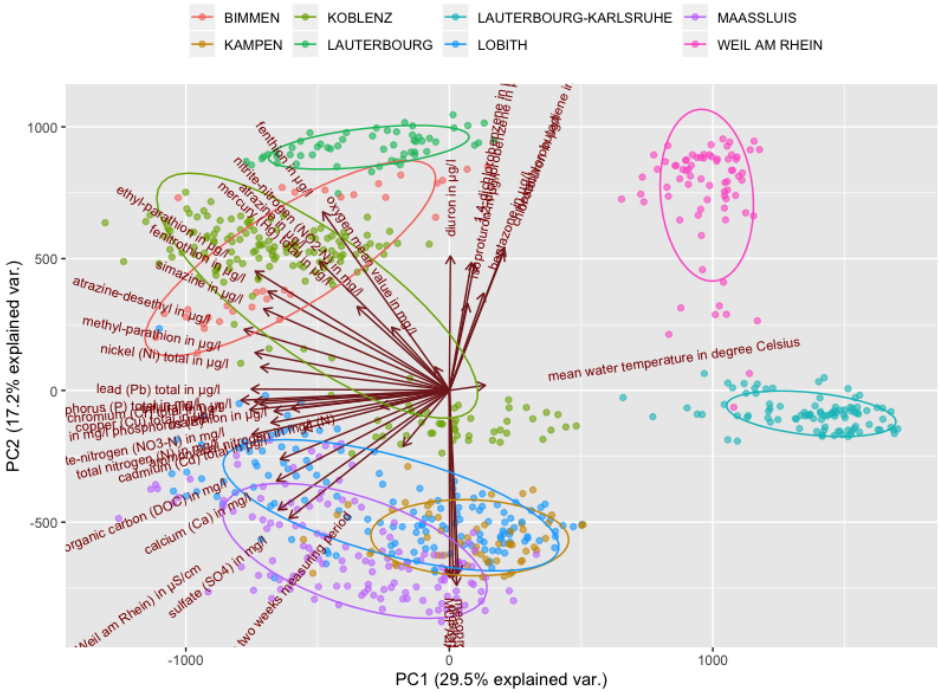


Figure 20: Biplot for the Rhine PCA (with monitoring location clusters)

The spatial location of the 8 retained monitoring locations drive the principal components. Because the samples per site (representing different measurements in time) are relatively closely clustered together, it can be observed that the temporal variability is smaller than the spatial variability.

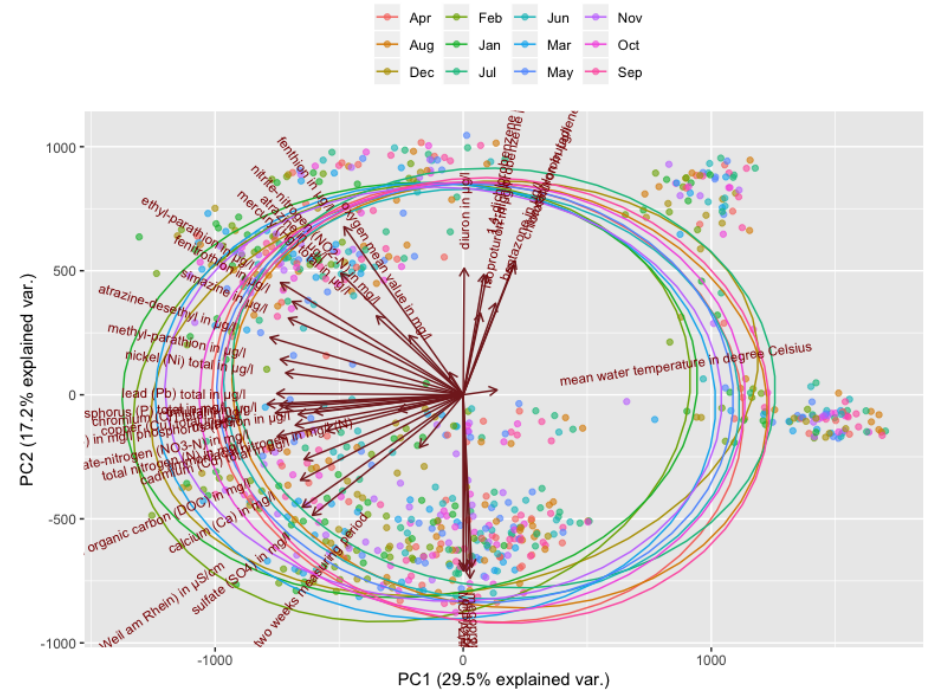


Figure 21: Biplot for the Rhine PCA (with monitoring location clusters)

There is no pattern observed with respect to the monitoring months. This indicates that the seasonal patterns (e.g. use of plant protection products in certain periods of the year) do not significantly change the observed mixture composition in the Rhine data set.

2.3. FRANCE

There are two datasets for France: one for the whole of France (referred to as France here) and one focused on the Adour-Garonne region (referenced as Adour-Garonne). Both were analysed with a PCA.

2.3.1. DATA PROCESSING

2.3.1.1. FRANCE

The original database is available as a long list in the long format i.e. per row individual measurements of chemicals. A first step was to aggregate this to 'samples' i.e. chemicals that were considered together as part of the same mixture.

- Chemicals that were measured on the same location and in the same month and year were considered part of the same sample.
- If multiple measurements of the same chemical were available per sample, the maximum of the values was taken.
- Measurements below the quantification limit were set to the reported quantification limit.

The dataset of France contains 311811 rows (samples) and 1,883 columns (chemicals measured). This dataset consisted of incomplete samples i.e. not all 1,883 chemicals were present in the samples. For the PCA analysis, a dataset with complete samples is required. Further data processing was done and a final dataset for analysis was prepared with 30,307 samples and 436 chemicals. Before PCA, the chemical concentrations were log-transformed and standardized.

2.3.1.2. ADOUR-GARONNE

The dataset consists of 87721 observations, which include 2408 sampling sites throughout 5 years from 2015-2019, and 409 variables.

As PCA requires a dataset with complete samples, pre-processing was applied to remove "empty cells", 3563 observations (with 290 sampling sites, 4 years from 2016-2019) and 77 chemicals retains in the final dataset – which is used for PCA analysis. The overall information of the final dataset can be illustrated as follow.

After pre-processing, the retained dataset mainly contains industrial substances (47%), following with pharmaceutical (21%), mixed-uses (15%) and argochemical substances (12%). Only a small part of dataset covers polyaromatic hydrocarbons (5%). A pie chart with the distribution of chemicals in final dataset is presented in Figure 22

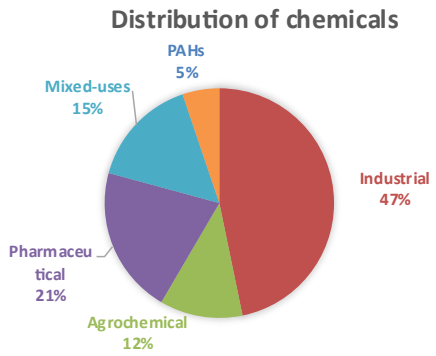


Figure 22: Distribution of chemicals retained in complete dataset of Adour-Garonne after pre-processing.

Spatial and temporal coverage of the retained dataset is illustrated in Figure 23. As can be seen from the figure, after pre-processing, only data from 2016 to 2019 is retained and data from 2019 is dominant thorough four years. The dataset does not equally cover all studied zones. Dordogne seems to be the most popular studied subject through four years, following by Charante. However, in 2019, Garonne covers the most data points. Côtiers aquitains et charentais is the zone that contains the least data point in the whole dataset.

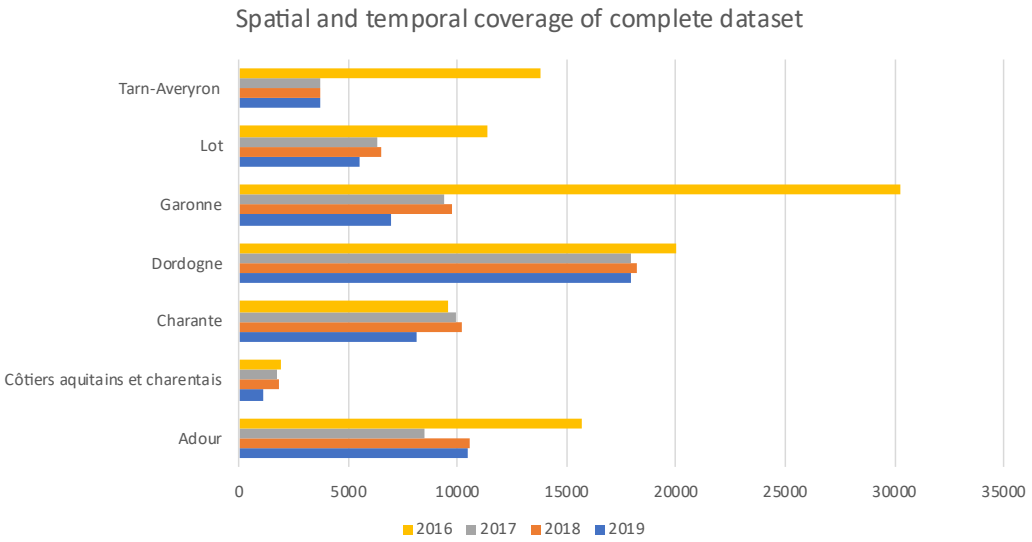


Figure 23: Spatial and temporal coverage of Adour-Garonne dataset after pre-processing.

2.3.2. OBSERVED MIXTURES

2.3.2.1. FRANCE

The PCA analysis explained a large part of the variance in the dataset: 78% of the variance was explained by the first three components. The biplot indicated a satisfactory PCA analysis, with clear clusters of sites and the chemicals showing clear correlations with the principal components (Figure 24). Because of the high number of chemicals in the dataset, the identified mixtures can be quite extensive. Therefore, only the 20 chemicals with the highest positive and negative Varimax scores per component are shown (Table 10).

In general, agrochemicals dominate this PCA. The varimax scores suggests that the first component separates agricultural chemicals and household, industrial chemicals and insecticides. For example, positive scores are found for the pesticides flutriafol, carbaryl, chlorsulfuron and monolinuron. No typical use pattern for these pesticides could be identified, they include herbicides, insecticides and fungicides. On the other side, chemicals are used in a variety of fields: Trichloropropane-1,2,3 is an industrial chemical (solvent, paint removal), dichlofluanide is used in wood protection, musk xylene is used in perfumes, acaricides to kill mites (e.g. propargite, fenpropathrin) and other insecticides (esfenvalerate) are also present.
The second component is dominated by (legacy) pesticides. Interestingly, no chemicals with significant negative scores were found for this axis, suggesting that (absence of) agricultural activity is the main driver for these mixtures. Solvents (bromoform, hexachlorobutadiene) are another group found positively correlated with this component. Many legacy (agro)chemicals

are found in this mixture e.g. DDT, hexachlorobenzene and hexachlorobutadiene, which are no longer used but persistent in the environment.
The third component includes agrochemicals both on the positive and negative side. This suggests that the two groups of agrochemicals are not used together.

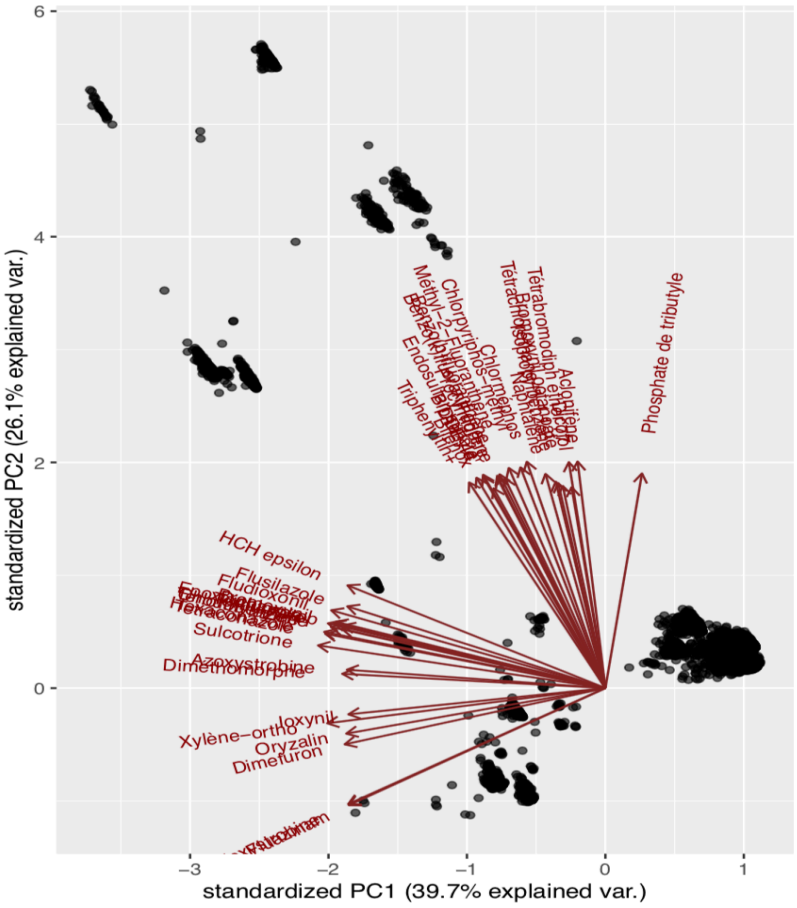


Figure 24: Biplot for the PCA for the France dataset.

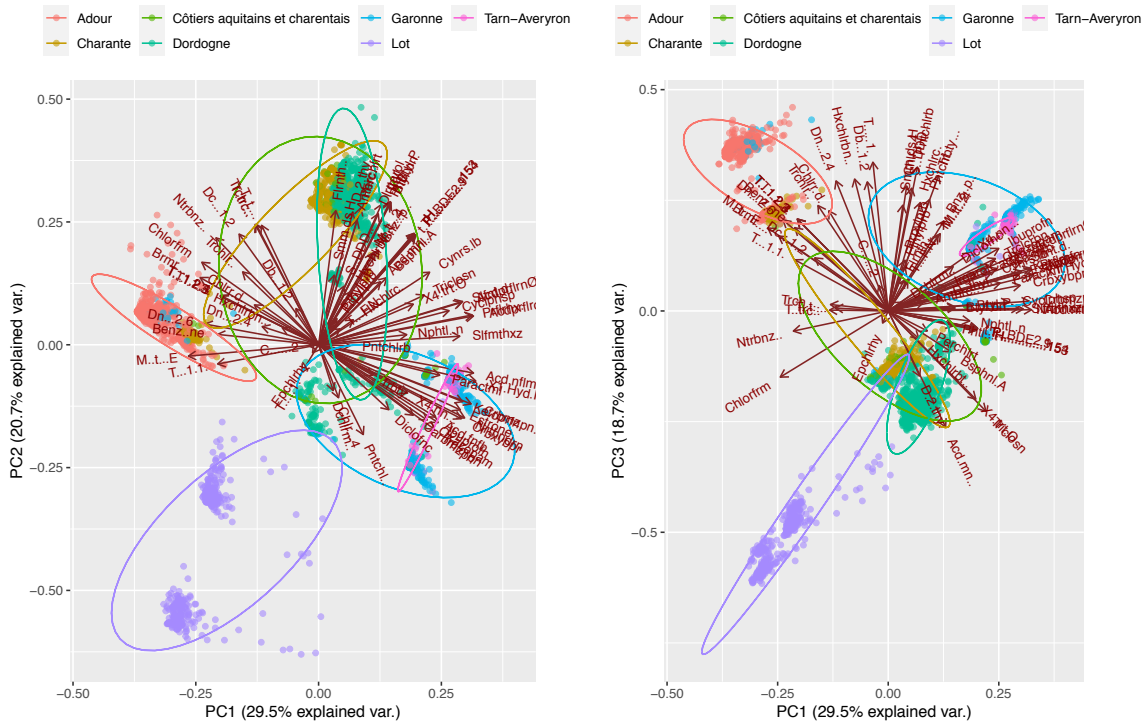
Table 10: Varimax loading scores for the three principal components of the France PCA. Only the top 20 positive and negative scores are shown.

Chemical	RC1
Flutriafol	0.99
Pyrazophos	0.99
Acetamiprid	0.99
Amétryne	0.99
Carbaryl	0.99
Carbétamide	0.99
Chlorsulfuron	0.99
Desmétryne	0.99
Flazasulfuron	0.99
Monolinuron	0.99
Paclobutrazole	0.99
Sulfosufuron	0.99
Mesosulfuron methyle	0.98
Bromuconazole	0.98
Picoxystrobine	0.98
Cyanazine	0.98
Florasulam	0.98
Sébuthylazine	0.98
Métoxuron	0.98
Monuron	0.98
Disulfoton	-0.85
Benfluraline	-0.86
Bioresméthrine	-0.86
Chinométhionate	-0.88
Bifenthrine	-0.88
Cyfluthrine	-0.9
Bromopropylate	-0.91
Tolylfluanide	-0.91
Musc xylene	-0.91
Tétradifon	-0.92
Fenpropathrine	-0.93
Acrinathrine	-0.93
Esfenvalerate	-0.93
Vinclozoline	-0.94
Thiométon	-0.96
Tefluthrine	-0.96
Oxadiargyl	-0.96
Captane	-0.97
Propargite	-0.98
Dichlofluanide	-0.99
Trichloropropane-1,2,3	-0.99

Chemical	RC2
DDT 24'	0.97
DDT 44'	0.97
Heptachlo epoxyde exo cis	0.97
Hexachlorobutadiène	0.97
Chloroforme	0.97
Bromoforme	0.97
Dichloroéthène-1,2 trans	0.97
Endosulfan B	0.97
Endosulfan A	0.97
Hexachlorobenzène	0.97
Isodrine	0.97
Chloronitrobenzène-1,4	0.97
p-octyl phénol	0.96
Dibutyltin+	0.96
DDD 44'	0.96
Trifluraline	0.96
Aldrine	0.96
Dieldrine	0.96
Endrine	0.96
DDD 24'	0.96

Chemical	RC3
Ethiophencarbe	0.97
Fluquinconazole	0.94
Penconazole	0.92
Desmediphame	0.92
Tebufenpyrad	0.92
Imazaquine	0.9
Nuarimol	0.89
Bromadiolone	0.88
Aldicarbe	0.88
Buprofézine	0.88
Hexachloroéthane	0.87
Ethoprophos	0.87
Bendiocarbe	0.87
Diéthofencarbe	0.86
Bupirimate	0.86
Flurochloridone	0.86
Cloquintocet-mexyl	0.86
Carfentrazone-ethyl	0.85
Dichlorométhane	0.83
Azaconazole	0.83
Pyrimiphos-éthyl	-0.49
pentabromodiph éther 85	-0.51
phosmet	-0.53
Dinitrotoluène-2,4	-0.54
Demeton-S-Methyl-Sulf.	-0.54
cis-1,3-dichloropropène	-0.55
trans-1,3-dichloropropène	-0.55
Chlorbufame	-0.58
Dibromoéthane-1,2	-0.61
Triallate	-0.67
Prométone	-0.68
Heptabromodiphényl éther	-0.68
Piperonyl butoxyde	-0.7
Chlorure de vinyle	-0.71
Flutolanil	-0.74
1-(3,4-diClPhyl)-3-M-urée	-0.75
Triadiméfone	-0.79
Fenpropidine	-0.8
Méthyl tert-butyl Ether	-0.82
Prophame	-0.91
Bitertanol	-0.93

The first three principal components (PCs) represent 68.9% of the variance – which is a satisfactory result of a principal component analysis. The biplots of PC1-PC2 and PC1-PC3 can be seen in Figure 25. Clusters of sampling points can be observed from the biplots. The observed separation is relative to sub basins, as being coloured in the figure. There is no trend/separation can be observed with respect to the temporal factors (year/month). The influence of spatial factor therefore can be seen as the most dominant role in the pattern observed from PCA results.



(a) (b)

Figure 25: Biplots of PCA results on Adour-Garonne dataset with colour code clustering for sub basins. Lines indicate in which area 95% of the sites fall. (a) PC1 - PC2; (b) PC1 - PC3

Varimax loadings give a quantitative indication for which chemicals are most correlated with the principal components. The Varimax loadings resulted from PCA on Adour-Garonne dataset is presented in Table 11.

Table 11: Varimax loading scores for the first three principal components of PCA resulted on Adour-Garonne dataset. Only the varimax scores > 0.4 (absolute value) are presented.

Substances	PC1	Group
Carboxyibuprofen	0,95	pharma
Ketoprofene	0,95	pharma
1-Hydroxy Ibuprofen	0,94	pharma
Carbamazepine epoxide	0,93	pharma
Estrone (E1)	0,93	pharma
Triclocarban	0,92	biocide,pharma
Norethisterone	0,91	pharma
Ibuprofen	0,91	pharma
Fenofibric acid	0,86	pharma
Niflumic acid	0,85	pharma
Oxazepam	0,84	pharma
(Perfluorooctanoic acid) PFOA	0,77	industrial
Perfluorohexanesulfonic acid	0,77	industrial
Carbamazepin	0,74	pharma
Paracetamol	0,74	pharma
Perfluorooctane sulfonate	0,7	/
Perfluorohexanoic acid	0,7	industrial
Sulfamethoxazol	0,69	pharma
Pentachlorophenol	0,66	PPP
4-Nonylphenol, branched	0,63	industrial
Tributyltin cation	0,61	/
Diclofenac	0,61	pharma
Cyclophosphamide	0,6	pharma
4-Chlorophenol	0,49	PPP, industrial
Free cyanide	0,46	/
1,2,4-Trichlorobenzene	-0,4	PPP, industrial
1,2,3-Trichlorobenzene	-0,4	PPP, industrial
1,3,5-Trichlorobenzene	-0,4	/
Methyl tertbutyl ether	-0,43	/
2,6-Dinitrotoluene	-0,47	industrial
Tetrachloroethylene	-0,53	industrial
1,2-dichloroethane	-0,54	industrial
Trichloroethylene	-0,55	industrial
Bromomethane	-0,6	/
Carbon tetrachloride	-0,61	industrial
Nitrobenzene	-0,84	industrial
Trichloromethane	-0,89	PPP,industrial

Substances	PC2	Type
Tetrabromodiphenyl ether (BDE-47)	0,94	PBDEs
Pentabromodiphenyl ether (BDE 99)	0,94	PBDEs
Pentabromodiphenyl ether (BDE 100)	0,94	PBDEs
Tribromodiphenyl ether (BDE 28)	0,94	PBDEs
Hexabromodiphenyl ether (BDE-153)	0,94	PBDEs
Hexabromodiphenyl ether (BDE-154)	0,94	PBDEs
Irgarol/Cybutryne	0,8	biocide
Dimethyl phthalate	0,64	industrial
Di-ethyl phthalate	0,6	/
Butyl benzyl phthalate (BBP)	0,59	industrial
Perchlorate	0,59	/
Dibutylphthalate	0,58	/
Chloroacetic acid	0,53	PPP, pharma
Di(2-ethylhexyl)phthalate (DEHP)	0,51	industrial
Triclosan	0,49	biocide,pharma
Total of hexachlorocyclohexanes	0,44	PPP
Delta-HCH (delta-hexachlorocyclohexane)	0,43	PPP
Octylphenol (4-(1,1',3,3'-tetramethylbutyl)-phenol)	0,42	PPP,industrial
Hexachlorobutadiene	0,41	PPP, industrial

Substances	PC3	Type
Hexachlorobenzene	0,94	PPP, industrial
2,4-Dinitrotoluene	0,87	/
1,1,2,2-Tetrachloroethane	0,86	/
Trichlorobenzenes (all isomers)	0,83	PPP,industrial
Dichloromethane	0,83	industrial
Pentachlorobenzene	0,81	PPP,industrial
Total of DDDpp, DDEpp, DDTop, DDTpp	0,8	PPP
1,2,4-Trichlorobenzene	0,77	PPP, industrial
1,2,3-Trichlorobenzene	0,77	PPP, industrial
1,3,5-Trichlorobenzene	0,77	/
Total of hexachlorocyclohexanes	0,74	/
Delta-HCH (delta-hexachlorocyclohexane)	0,74	PPP
2,6-Dinitrotoluene	0,72	industrial
Tributyltin cation	0,68	/
Chloroethene (vinylchloride)	0,66	/
4-Chlorophenol	0,65	PPP, industrial
Benzene	0,63	PAH
Methyl tertbutyl ether	0,63	/
Bromomethane	0,61	/
Pentachlorophenol	0,53	PPP
Benzo(a)pyrene	0,49	PAH
1,2-dichloroethane	0,46	industrial
4-methyl-phenol	0,42	/
2-methyl-phenol	0,42	/
Octylphenol (4-(1,1',3,3'-tetramethylbutyl)-phenol)	-0,62	PPP,industrial
Triclosan	-0,7	biocide,pharma
Chloroacetic acid	-0,71	PPP, pharma

The first component is dominated by pharmaceutical substances and a few industrial/PPP substances. Frequently used medicines as pain killers, hormonal pills, anti-inflammatory drugs are observed the most in the first PC. The explanation for distribution/co-occurance of these substances is discussed in 2.3.3. The negative varimax scores indicate a mix of chemicals mainly used in agricultural and industrial sectors. The analysis indicates that chemicals on positive side of PC1 do not co-occur with ones on negative side. The second and third PCs are mixtures of industrial/agricultural substances.

2.3.3. INTERPRETATION

2.3.3.1. FRANCE

To better understand why certain chemicals co-occur, spatial factors of the sites were taken into account. For each of the sites, information is available on the region they belong to (Figure 26). This analysis is similar to the analysis with the Waterbase dataset, which only contained sites from France in the final dataset. Therefore, we compared the results per Région with the previous analysis. Looking at the number of sites per Région (Table 12), Bretagne, Pays de la Loire and the sites unassigned to a Région were underrepresented (<50 sites) which prevented a good interpretation of these sites.

Table 12: Number of sites per Région and river basin in the processed France dataset.

REGION	SITES
AUVERGNE-RHONE-ALPES	4,902
BOURGOGNE-FRANCHE-COMTE	6,050
BRETAGNE	54
CENTRE-VAL-DE-LOIRE	662
CORSE	471
GRAND-EST	4,350
HAUTS-DE-FRANCE	2,305
ILE-DE-FRANCE	2,660
NORMANDIE	3,696
OCCITANIE	2,276
PAYS-DE-LA-LOIRE	6
PROVENCE-ALPES-COTE-D'AZUR	2,831
UNASSIGNED	44

Compared to the Waterbase dataset, the different Régions overlapped more. Normandie and to a lesser extent Hauts-De-France clusters were situated more to the left compared to the other Régions. These are regions with old industry. Other heavily industrialized Régions which had similar chemical concentrations in the Waterbase PCA (Centre-Val-De-Loire, Grand-Est, Ile-de-France), are no longer found together with these two Régions. Possibly, the higher relative weight of agrochemicals in the France dataset caused this shift. The other Régions could not be clearly distinguished from one another. Looking at the biplot with the first and third principal component, the distinction between Occitanie, Provence-Alpes-Côte-d'Azur and Bourgogne on one side and the other Régions (e.g. Grand-Est) becomes apparent again, likely associated with their typical agricultural and industrial land use.

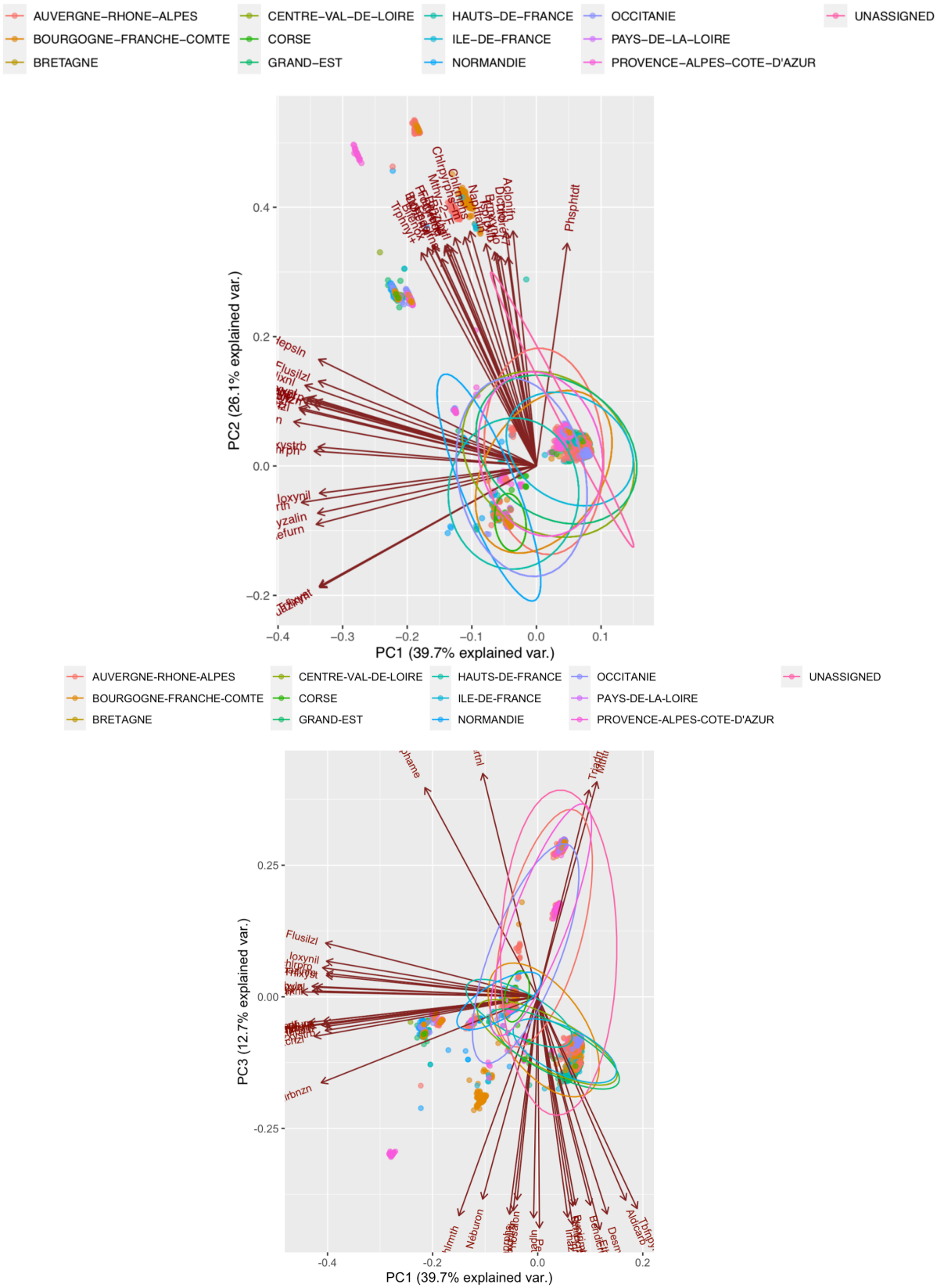


Figure 26: Biplot of the France PCA, with clustering per Région. The biplots for PC1 and PC2 (top) and PC1 and PC3 (bottom) are shown.

From PCA resulted on Adour-Garonne dataset, five mixtures of chemicals were observed. Except the first mixture (positive side of PC1), other four mixtures cover a broad group of chemicals. There is no immediate explanation for their co-occurrence yet and more investigation is required to go further to interpret this result. On the other hand, the mixture dominating positive side of PC1 mainly contains pharmaceutical substances. This mixture is helpful for us to understand factors having impacts to its occurrence.

Score plot of PC1-PC2 is clustered into three groups as in Figure 27. Orange cluster consists of sampling points which have highest presence of pharmaceutical substances in samples. Blue cluster consists of sampling points which have lowest presence of pharmaceutical substances in the samples. Grey cluster is in between orange and blue clusters.

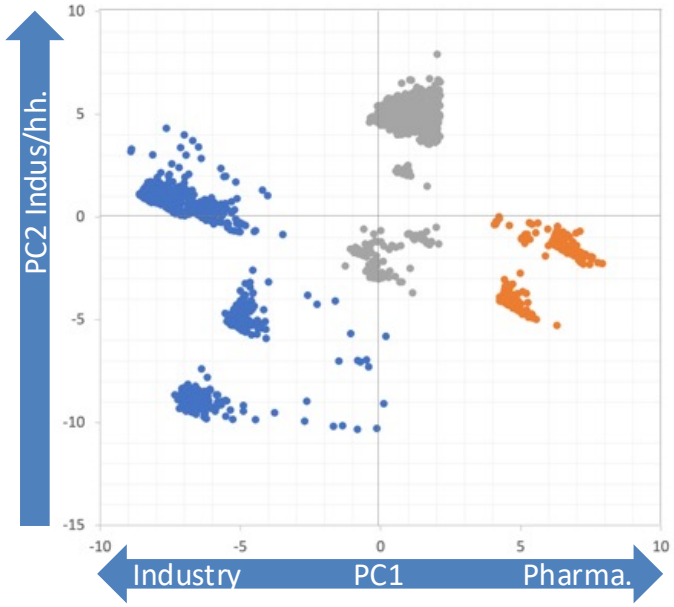


Figure 27: Score plot PC1-PC2 of PCA resulted on Adour-Garonne dataset. Three clusters are defined based on PC1.

The orange and blue clusters are presented in the Adour-Garonne map to understand further their pattern, as illustrated in Figure 28. Location of pharmaceutical factories is also marked in the map.

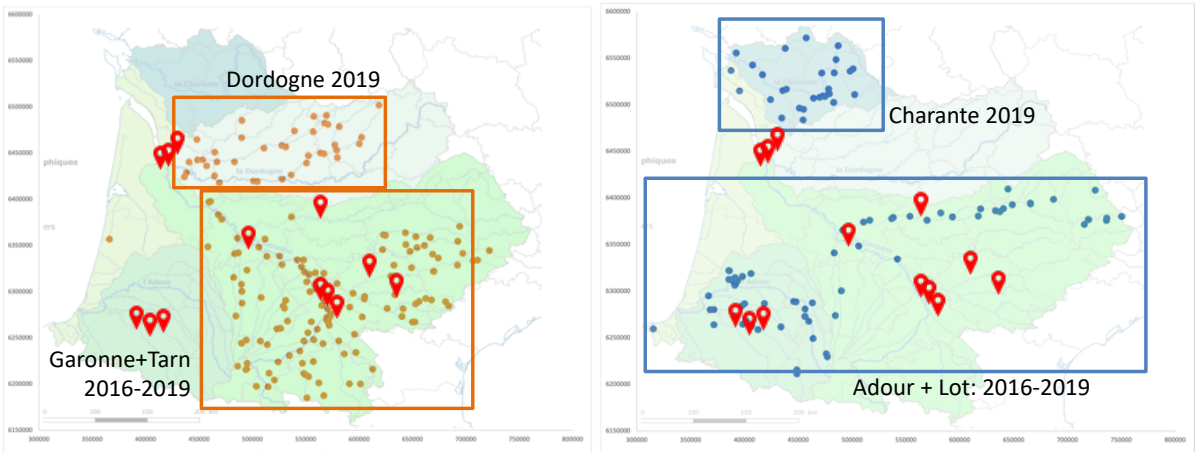


Figure 28: Spatial distribution of orange and blue cluster on the Adour-Garonne map. Red check-in points represent location of pharmaceutical factories.

As can be seen from the figure, sampling points with high presence of pharmaceutical substances are distributed mainly in Garonne and Tarn during the whole investigated time period (2016-2019). Whereas the blue sampling points are mainly present in Adour and Lot from 2016 to 2019. Dordogne and Charante are also a part of these two clusters, however, pattern is only observed for 2019.

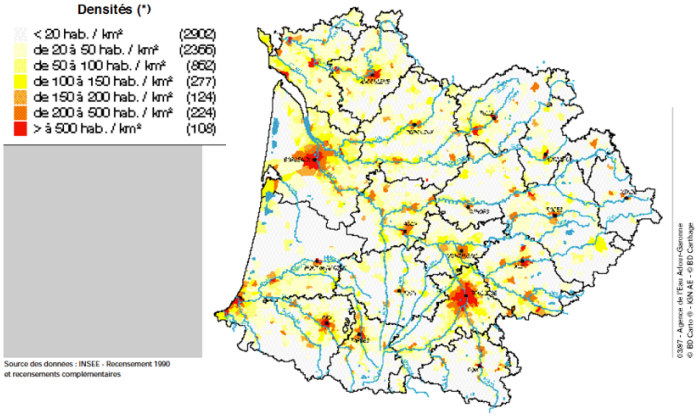


Figure 29: Population density of Adour-Garonne[3]

No direct link between production plants and the exposure pattern can be observed. The exposure of pharmaceutical substances in this region seems not to have correlation with production sites. Investigation is therefore focused on different factors: use-pattern and spatial factor. Population density of the region is shown in Figure 29. Garonne and Tarn are both highly dense in population with Toulouse is a metropolitan, whereas Adour and Lot are less dense. The exposure of pharmaceutical substances seems to correlate with the population density. This can be explained that these drugs are commonly used and their presence in surface water might be due to the residue from human waste. A study from Destrieux et al. (2017) on exposure of drug residues in Toulouse shows that many of pharmaceutical substances can still be quantified at the downstream of wastewater treatment plants (WWTP). However, the concentration of these chemicals is not significantly greater than upstream of WWTP. Their conclusion is that WWTPs have negligible contribution to the presence of drugs in surface water and the upstream watershed is considered as the main source.

Beside population, geographical factor also needs to be considered. Water from Lot subbasin is originated from Massif Central. It consists of two major catchments Truyère and Lot and a few small catchments along the main river. Water catchments in this area is considerably smaller

[3] INSEE – Recensement 1990 et recensements complémentaires.

than others in Adour-Garonne. The water from Lot sub basin then joins in Garonne River. This area is known as a natural reserve zone, which is less exposed to artificial infrastructure.
Water of Adour sub basin is originated from Pyrenée mountains. Its water catchments do not join in Garonne River but flow directly to Bay of Biscay (Atlantic Ocean).
On the other hand, Garonne and Tarn sub basins consist of dense water catchments. All the catchments merge together along the downstream of Garonne River. The water in these two sub basins collect water from other zones. In another word, these regions can be considered as "downstream zones" – which are more diluted and polluted. This might be also a reason that the presence of drugs in these two sub basins is higher than other zones.

In brief, use-pattern from population density and location of sampling points seem to play important role in the distribution of chemicals in Adour-Garonne, especially pharmaceutical substances. Production sites seem not to have correlation with the exposure of chemical in Adour-Garonne.

2.4. SUMMARY OF OTHER DATABASES

Only a brief summary of the following databases is reported here: The Netherlands (Dutch monitoring database, from Waterkwaliteitsportaal), Flanders (monitoring database by the Flemish Environmental Agency VMM) and the database from the third Joint Danube Sampling campaign (JDS3).

2.4.1. THE NETHERLANDS (WATERKWALITEITSPORTAAL)

The database contains 42,752 samples with 1,169 chemicals measured in total. Data processing for the PCA resulted in a dataset with 4,568 samples and 89 chemicals. The database is mainly focused on agrochemicals and hence the mixtures are less representative.

The PCA was able to explain a large part of the variance: 80% of the variance was captured by the first three principal components. The first component is marked by a large mixture of agrochemicals on one side and a smaller mixture of 4 agrochemicals on the other side i.e. diuron (herbicide), bitertanol (fungicide), iprodione (fungicide) and fipronil (insecticide). The second component similarly distinguished a wide mixture of agrochemicals on one side and a small mixture of 4 agrochemicals on the other side i.e. pymetrozine (insecticide), thiofanaat-methyl (fungicide) and chlortoluron (herbicide). The third component shows one mixture of six chemicals to be relevant i.e. imidacloprid (insecticide), azoxystrobin (fungicide), metribuzin (herbicide), ethofumesaat (herbicide), clomazon (herbicide) and prosulfocarb (herbicide). An explanation why these agrochemicals co-occur is not immediately apparent. Possibly they are used in similar fields or have similar uses e.g. as garden chemicals versus large-scale agricultural use. The biplot clustering per water body suggests that there are differences between water bodies, but these were not further studied in detail.

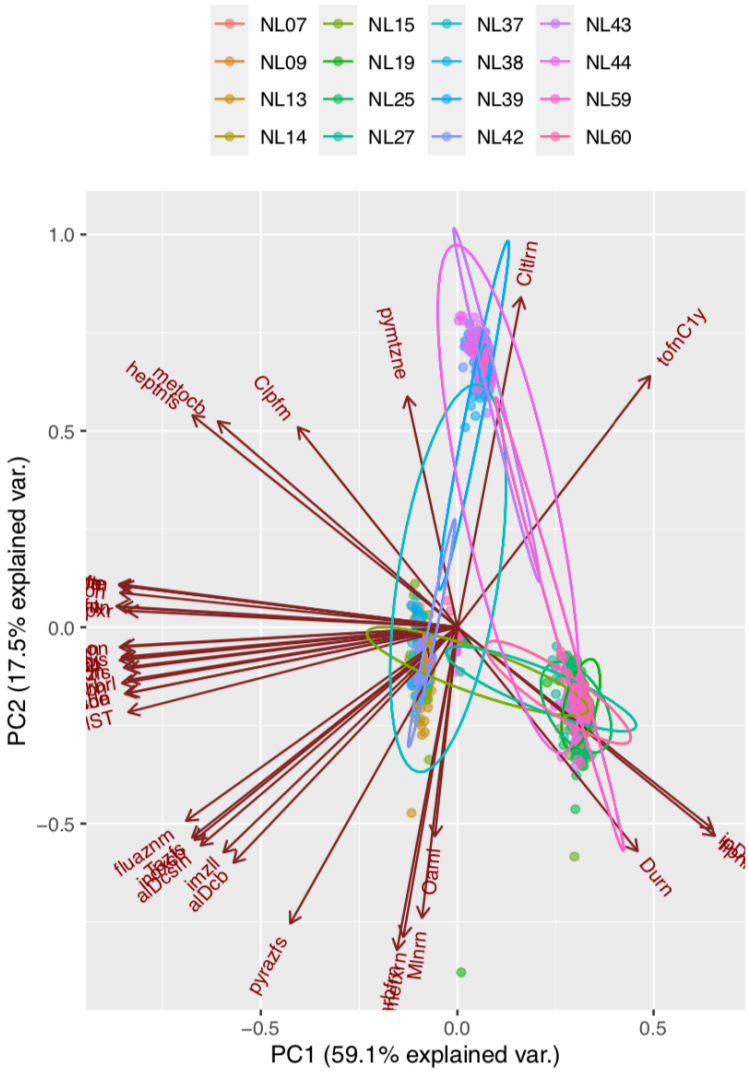


Figure 30: Biplot for the PCA for the database of Waterkwaliteitsportaal (The Netherlands). Clustering is visualized per water body.

2.4.2. FLANDERS (VMM)

The dataset contains over 180 chemicals, covering agrochemicals, PAHs and metals. The PCA explained a reasonable amount of the variance i.e. 41% of the variance was explained by the first three components.

The first component is largely determined by PAHs, suggesting this pollution is strongly linked to PAH emissions. The second component is correlated with a large number of (legacy) pesticides and a few metals (As and V). Agriculture seems to be the main land use responsible for this component. Lastly, the third component is associated with metals (Zn, Ni, Co, Cd) and detergents (e.g. nonyl phenol) and negatively associated with the herbicide desethylatrazine, suggesting industry and households are mainly responsible for this co-exposure.

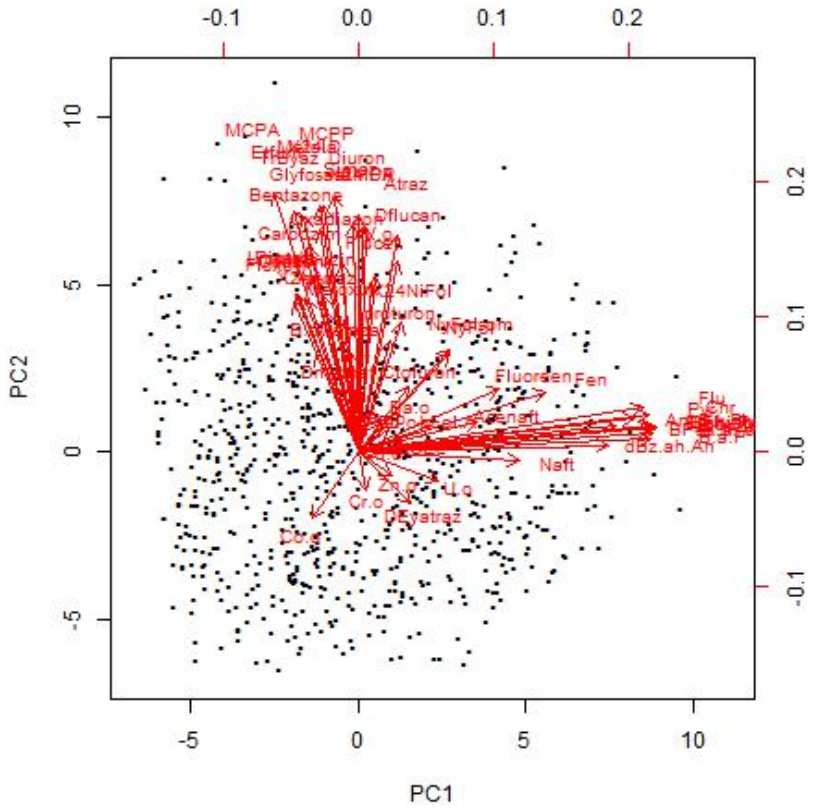


Figure 31: Biplot for the PCA on the Flanders dataset.

2.4.3. DANUBE (JDS3)

The database from the Danube sampling campaign consists of a large number of chemicals measured (395 in total) but only a small number of sampling occasions (191). After data processing, a dataset with 44 samples for 248 chemicals was retained. The large number of chemicals for a small number of samples hindered the PCA, resulting in only a small amount of the variance (20% by the first two components) being explained by the PCA. Hence, the outcome of the PCA should be interpreted with care.

The database contains many pharmaceuticals, and these are also most significant for the first principal component. A wide mixture of pharmaceuticals, e.g. clonazepam, verapamil, biocide, triazolam, chlorprothixene and diazepame, and the biocide cybutryne are contrasted with a mixture of 8 chemicals i.e. the pharmaceuticals nordiazepam, hydrocodone, (dihidro)codeine, Benzoylecgonine, the artificial sweetener sucralose and the metals copper and arsenic. The second component contrasts a mixture of pharmaceuticals and household chemicals (e.g. sucralose) with several water chemistry parameters (e.g. Na, Mg, DOC) and pharmaceutical (e.g. pipamperone, trimethoprim).

The biplot with clustering per country suggests that the chemical cocktails can be different per country, but the number of samples is too low to draw any strong conclusions about the influence of e.g. land use or downstream vs upstream.

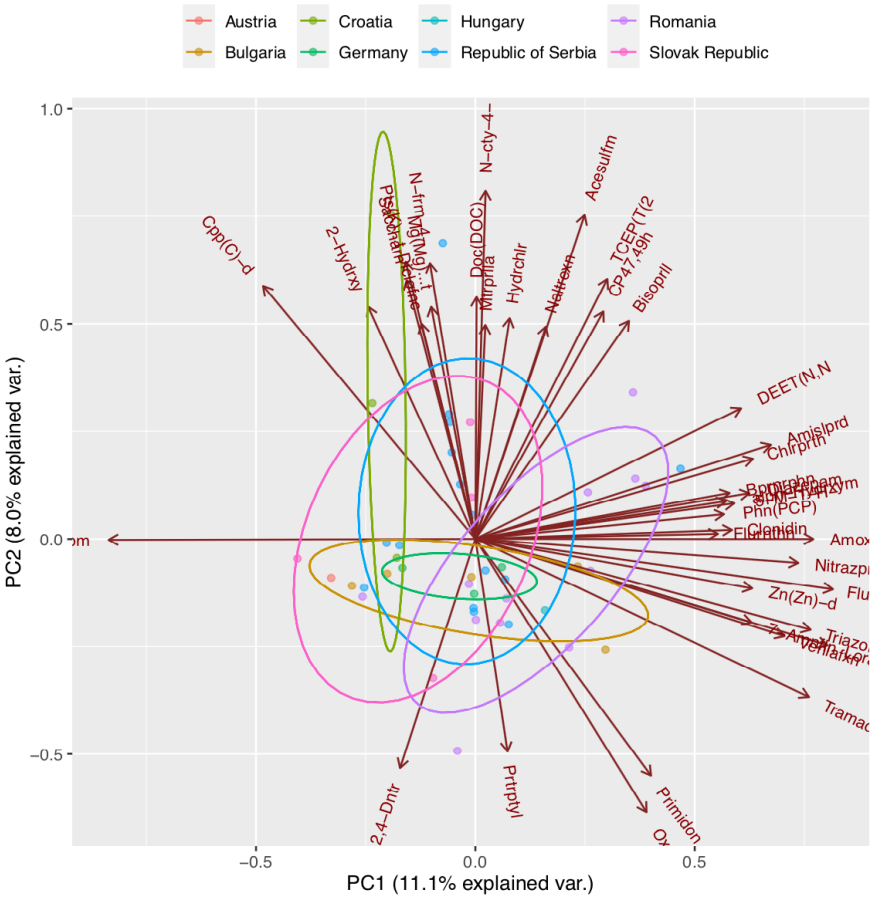


Figure 32: Biplot for the Danube dataset PCA, with clustering per country.

3. TECHNICAL ANNEX C: ENVIRONMENTAL MIXTURE RISK IN DATABASES

For the cumulative risk assessment, the focus was made on those datasets supporting the identification of mixtures of concern for cumulative risk and/or ecological risk (risk identified) which would merit a review using the approach of the CEFIC MIAT decision tree for assessing effects from exposures to multiple substances. For the cumulative risk assessment, two databases were selected: i.e. the Eau-France – Adour/Garonne region and the Rhine database. The Eau-France Adour/Garonne region represents a dataset with samples covering a large area and substances from different sources which are covered by different legislations. The Rhine database represents a hot-spot example, with a limited number of sampling points (Table 13).

Table 13: Summary of mixture exposure and analytes covered in the different databases used in the environmental mixture risk assessment.

Database	Number of samples covered	Number of analytes
Rhine	394	51
Adour-Garonne	3563	77

3.1. WATERBASE

Rodea-Palomares et al. (in preparation) have done an extensive analysis of the Waterbase, calculating mixture risk using toxic units and applying the CEFIC MIAT decision tree. Therefore, we have chosen not to repeat this analysis but use their findings in our discussions.

3.2. RHINE

3.2.1. ASSESSMENT OF MIXTURE RISKS

Figure 33 (upper panel) shows the cumulative risk in the Rhine dataset under the more realistic scenario (dealing with non-detects see section 1.2.2). The 10^{th} to 90^{th} percentile of the HI, ranged between 0.10 and 3.6, with a median HI of 1.1 (Table 14). The distribution of the mixture exposures over the different mixture groups is as follows: 19% are classified as Group I, 46% as Group II, 12% to Group IIIa and 22% to Group IIIb.

Under the unrealistic worst-case scenario, the cumulative risks were substantially higher: the 10th to 90th percentile of HI, ranged between 1.9 and 7.2, with a median HI of 4.9 (Table 15, Figure 33 lower panel). 73% of the mixture exposures belong to Group I, indicating the exceedance of the HC5 of at least one substance. 23% of the mixture exposures belong to Group IIIb, the more complex mixture category. The remaining mixture exposures are categorized in either Group II (2%) or Group IIIa (2%).

Hence, unrealistic worst-case scenario would indicate a concern for environmental risks for 98% of the mixture exposures (i.e. belonging to either Group I, Group IIIa or Group IIIb), while in the more realistic scenario this is the case for 50% of the mixture exposures there is a concern for environmental risks. However, it should be noted that a relatively simple refinement of the threshold level for one of the driving substances, reduces these risks significantly.

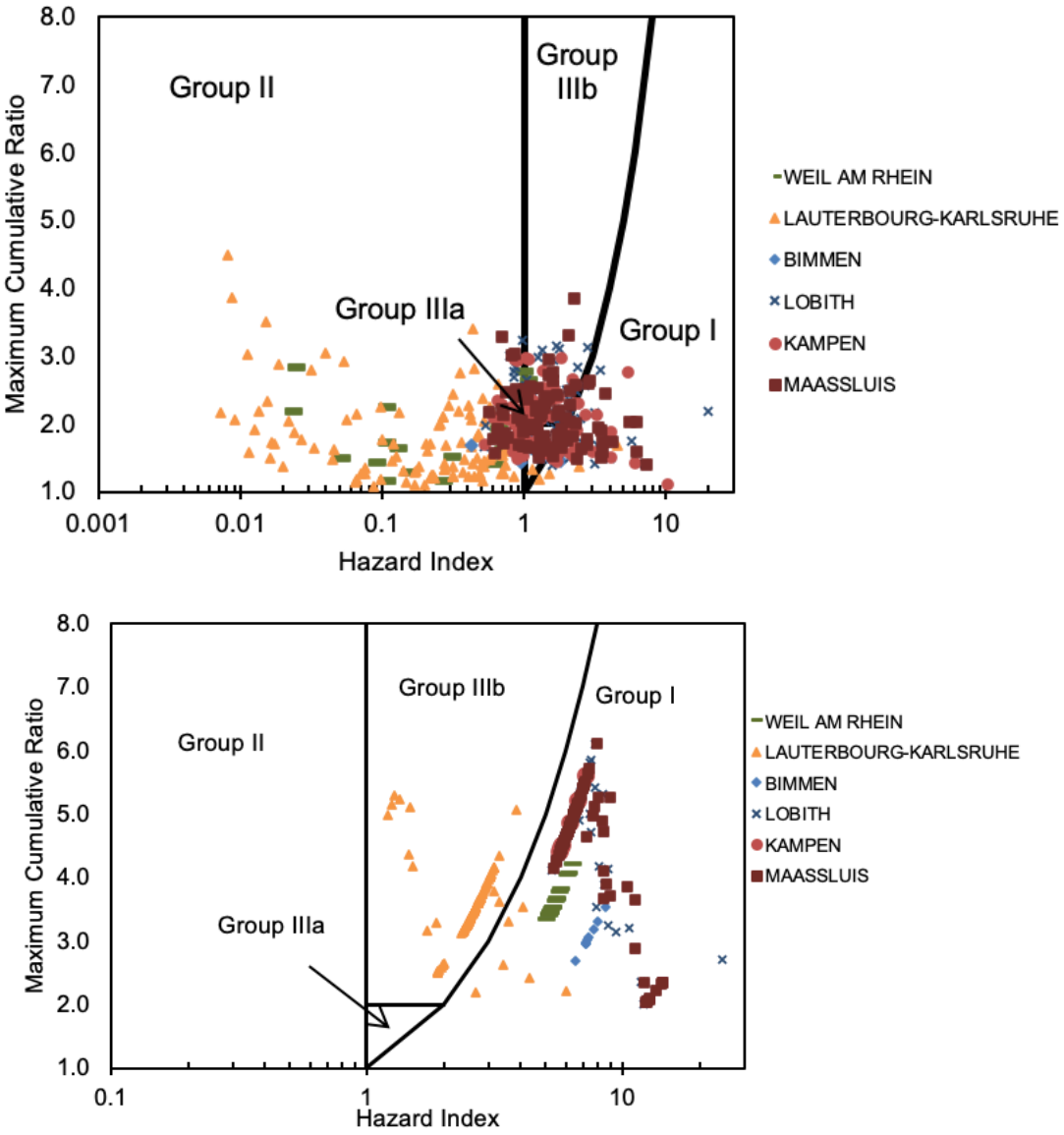


Figure 33: Plot showing the Maximum Cumulative Ratio as a function of the Hazard Index for the Rhine dataset for the more realistic scenario (top, showing cumulative risks of the detects only) and the unrealistic worst-case scenario (bottom, showing cumulative risks of the detects at the measured concentration and of the non-detects at the detection limit). Different symbols indicate different sampling sites.

Table 14: Overview of statistics for the Hazard Index for the mixture exposures.

	More realistic scenario (<DL=0)	Unrealistic worst-case scenario (<DL=DL)
Minimum HI	<0.01	0.68
Maximum HI	19.8	23.5
Median HI	1.1	4.9
10th percentile	0.10	1.9
90th percentile	3.6	7.2

Table 15: Overview of the percentage of mixture exposures belonging to the different mixture groups for the Rhine.

	More realistic scenario (<DL=0)	Unrealistic worst-case scenario (<DL=DL)
Group I	19%	73%
Group II	46%	2%
Group IIIa	12%	2%
Group IIIb	22%	23%

Allocating chemicals to the different chemical legislations is difficult because some chemicals are subject to multiple chemical legislations (e.g. copper is a REACH substance, a biocidal active substance, a plant protection product, micronutrient in fertilizer, etc…), some chemicals are legacy chemicals (these are no longer used but there is still historical pollution, e.g. nonylphenol), some chemicals are both REACH chemicals as well as degradation or combustion product (e.g. PAHs). When chemicals are -grosso modo- split up over the different chemical legislations, REACH chemicals seem to drive the mixture risks in the Rhine data set. The main monitoring locations are located in or downstream the Ruhr area which is a heavenly industrialized region in Germany. The 10^{th} to 90^{th} percentile of HI for the REACH chemicals ranges between 0.05 and 3.5, with a median HI of 1.0. Compared to this all other substances (mainly PPP) only contribute minorly, with the 10^{th} to 90^{th} percentile of HI ranging between <0.01 and 0.41, and a median HI of 0.07. It should be noted that the contribution of the REACH chemicals is an overestimation of the actual REACH contribution. This is because this analysis did not differentiate mixture risks if a substance is listed under different chemical legislations, historical pollution and combustion products as described above.

The driving chemicals under the more realistic scenario (with respect to detection limits) are listed in Table 16 and Table 17. Over the entire dataset, it is mainly the PAH benzo(ghi)perylene and zinc that drive the cumulative risks. In the Group I mixtures, an HQ>1 is observed for 70 and 11 of the mixture exposures for benzo(ghi)perylene and zinc, respectively. For the Group IIIa mixtures, again the PAH benzo(ghi)perylene and zinc are the driving chemicals, with an HQ>0.5 observed in, respectively, 45 and 3 of the mixture exposures, while copper is popping up as the driving chemical in only one of Group IIIa mixtures. In the complex mixtures (Group IIIb), benzo(ghi)perylene and zinc are again the main contributors. However, also the pesticides isoproturon, diazinon and diuron, the metals copper, chromium and nickel and the PAH benzo(b)fluoroanthenene contribute to some extent to the overall mixture pressure (i.e. HQ>0.1 in at least one mixture exposure). The pesticides isoproturon, diazinon and diuron are currently not approved for use within the EU. Most of the driving chemicals are also listed as priority substances under the WFD (chromium, nickel, isoproturon, diuron, benzo(ghi)perylene), while others are also known as 'legacy' chemicals. Malaj et al. (2015) also noted that pesticides and PAHs are among the most contributing substances to chemical risks associated with organic chemicals in Europe. Diuron and diazinon have been identified as being among the most critical pesticides over 5 river catchments in Switzerland (Moshet et al. 2014), while isoproturon has been noted to be one of the substances driving mixture risks in European surface waters (Price et al. 2012). Within the SOLUTIONS project, diuron has been reported to drive predicted mixture risks for algae in the Rhine river, while the metals copper, nickel and zinc and the PAH benzo(ghi)perylene are driving mixture risks for invertebrates in the Danube river (Backhaus et al. 2018). Within the same project, diazinon has been identified for driving mixture risk for invertebrates and algae in wastewater treatment plant effluents in the German river Holtemme (Beckers et al. 2018). Metals have been observed to drive mixture pressure in several studies: e.g. Price et al. (2012) reported that

metals drive mixture risks in Group I and Group IIIb mixtures in European surface waters and wastewater treatment plant effluents in the UK. Metals have been reported to drive predicted mixture risks for fish and invertebrates in the Danube river. It should be noted that none of these studies and also the present study did not take bioavailability effects into account. It can be expected that incorporating bioavailability normalization into the HC5 derivation, via for instance the biotic ligand models, the predicted cumulative risks would decrease compared to the current analysis. However, even after bioavailability normalization substantial risks can be predicted in 'hotspot' water bodies in the with the HI/HQ approach, such as in the Dommel in the Netherlands (Nys et al. 2018).

The driving chemicals under the unrealistic worst-case scenario (with respect to detection limits) are listed in Table 17. For ethyl-parathion and diazinon the HQ>1 in approximately 70% of the mixture exposures, while for benzo(ghi)perylene and zinc a HQ>1 was observed for 27% and 8% of the exposures. It should be noted that for parathion and diazinon measured concentrations were below the detection limit in 100% and 84% of the mixture exposures.

Table 16: Substances driving the cumulative risks for each of the mixture groups in the Rhine dataset under the more realistic scenario.

Mixture group	Substance	Maximum HQ	Number of exposures where mixture		
			HQ>1	HQ>0.5	HQ>0.1
Group I (*n=75*) (only substances for which HQ in at least one mixture exposure is >1)	benzo(ghi)perylene	9.06	70	71	71
	zinc (Zn) dissolved	1.61	11	24	59
	diazinon	1.08	1	1	2
	benzo(b)fluoranthene	3.10	1	3	60
	indeno(1,2,3-cd)pyrene	4.68	1	1	13
	benzo(k)fluoranthene	1.55	1	1	15
	Ethyl-parathion	1.45	1	1	1
Group IIIa (*n=49*) (only substances for which HQ in at least one mixture exposure is >0.5)	benzo(ghi)perylene	0.97	0	45	46
	zinc (Zn) dissolved	0.92	0	3	14
	Copper (Cu) dissolved	0.72	0	1	48
Group IIIb (*n=86*) (only substances for which HQ in at least one mixture exposure is >0.1)	benzo(ghi)perylene	0.99	0	62	83
	zinc (Zn) dissolved	0.89	0	10	72
	isoproturon	0.74	0	3	31
	copper (Cu) dissolved	0.59	0	2	86
	chromium (Cr) total	0.78	0	1	34
	diazinon	0.51	0	1	2
	nickel (Ni) dissolved	0.17	0	0	3
	benzo(b)fluoranthene	0.15	0	0	10
	diuron	0.12	0	0	1

HQ=Hazard Quotient

Table 17: Driving chemicals under the more realistic scenario (i.e. detects at measured concentration + non-detects set at detection limit) and percentage of samples with a HQ>1, HQ>0.5 and concentration below DL.

	HQ>1	HQ>0.5	Concentration <DL
ethyl-parathion	71%	71%	100%
diazinon	67%	72%	84%
benzo(ghi)perylene	27%	82%	34%

HQ= hazard quotient, DL= detection limit

3.2.2. RELEVANCY OF THE SELECTED ENVIRONMENTAL EFFECT LEVELS

While performing cumulative risk assessment, it is crucial that the selected effect data are representative for the assessments performed. To allow a high-throughput analysis in the current assessment, HC_5-values have been calculated based on parameters selected from the database published by Posthuma et al. (2019). The SSDs behind these HC_5s are associated with different levels of uncertainty (and associated extrapolation factors). Table 18 shows the data-quality label for the SSDs of the different substances driving the mixture pressure assessment as reported by Posthuma et al. (2019). For most of the substances, the chronic SSD is based on chronic NOEC/EC_{10} data covering at least 10 species. However, for benzo(ghi)perylene, chromium and nickel chronic SSDs have been extrapolated based on acute NOECs or EC_{50}s (incorporating an extrapolation factor of 3 and 10, respectively).

Due to the extent of the dataset (covering over 12000 substances), the toxicity data underlying the SSD-parameters may not have undergone the same data quality scrutiny assessment as in the substance-specific effect assessments for regulatory compliance assessment, such as in the REACH framework. For the driving chemicals (see Table 17), the relevancy of the selected HC_5-values has been evaluated. Comparison has been done with freshwater PNECs published on the ECHA dissemination website. For PAH, for which no PNEC has been submitted under the REACH registration dossier, chronic PNECs were extracted from the PETROTOX-tool, which calculates calculated PNECs based on the Target Lipid Model (Redman et al. 2017). For the pesticides listed Table 16, comparison of the HC_5 was made to the AA-EQS of the WFD (EC 2013/39/EU).

Table 18: Overview of data quality of SSDs underlying the used HC_5 for mixture pressure assessment for substances driving the HI/HQ approach.

Data quality description in Posthuma et al. (2019)	Substances
Chronic NOEC not extrapolated - Officially enough species (>10) for ERA with SSDs	Isoproturon, ethyl-parathion, zinc, diazinon, copper, benzo(b)fluoranthene, benzo(k)fluoranthene,
Chronic NOEC extrapolated from Acute NOEC - Officially enough species (>10) for ERA with SSDs	Benzo(ghi)perylene
Chronic NOEC extrapolated from Acute EC50 - Officially enough species (>10) for ERA with SSDs	Chromium, Nickel

The HC_5 calculated from the database of Posthuma et al. (2019) is in reasonable agreement with the PNEC registered under REACH for metals (all within 3-fold difference) and with the WFD AA-EQS for the pesticides (all within 2-fold difference). However, for the PAHs the selected HC5-values systematically overestimates toxicity compared to the PNEC derived from the Target Lipid Model (TLM): up to 100-fold difference is observed. Especially for the PAH benzo(ghi)perylene, a large overestimation of the effect threshold was observed. Given that PAHs, and especially benzo(ghi)perylene were identified as one of the driving chemicals and the dependency of the method on the selected effect values, the HI/HQ approach is further refined using the TLM-derived PNECs for the PAHs.

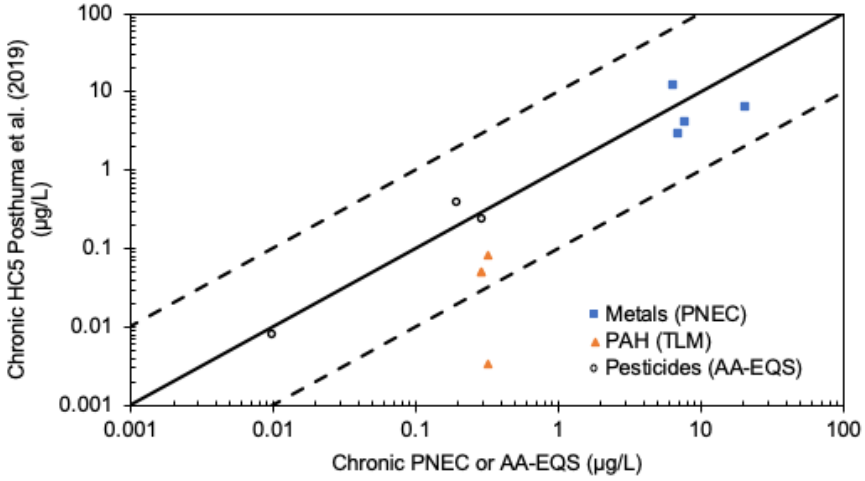


Figure 34: Comparison of calculated HC5 values based on SSD-parameters selected from Posthuma et al. (2019) and reference environmental threshold values for the driving chemicals in the Rhine dataset. For metals, HC5 values are compared to the chronic freshwater PNEC values from the ECHA dissemination website (blue squares, consulted May 2021). For the polycyclic aromatic hydrocarbons (PAH), HC5 values are compared to the chronic freshwater PNEC calculated with the Target Lipid Model (Redman et al. 2017, red triangles). For the pesticides, HC5 values are compared to the annual average-environmental quality standard from the Water Framework Directive (EC 2013/39/EU, open circles). The full line represents a perfect match between HC5 and PNEC or AA-EQS, the dashed lines represent a 10-fold difference.

3.2.3. REFINING THE HI/HQ APPROACH WITH TLM DERIVED PNECS FOR PAH

Figure 35 shows the cumulative risk in the Rhine dataset for the more realistic scenario with the refined effect thresholds for PAHs. This refinement resulted in a considerable decrease of the mixture pressure, e.g. median HI was decreased by 2.2-fold to 0.51 (Table 19). The distribution of the mixture exposures over the different mixture groups did also change accordingly with most mixture exposures belonging to Group II, and only a small fraction of the mixtures belonging to Group IIIa (2.5%) and Group IIIb (10.2).

While PAHs where identified as important drivers in the previous assessment, this pictures changes totally when using the refined PNEC values for PAHs in the HI/HQ assessment. In all mixture groups of concern, metals (Zn, Cu and/or Cr) pop up as the driving chemicals with also some contributions of the EU-banned pesticides isoproturon, diazinon, diuron and ethyl-parathion.

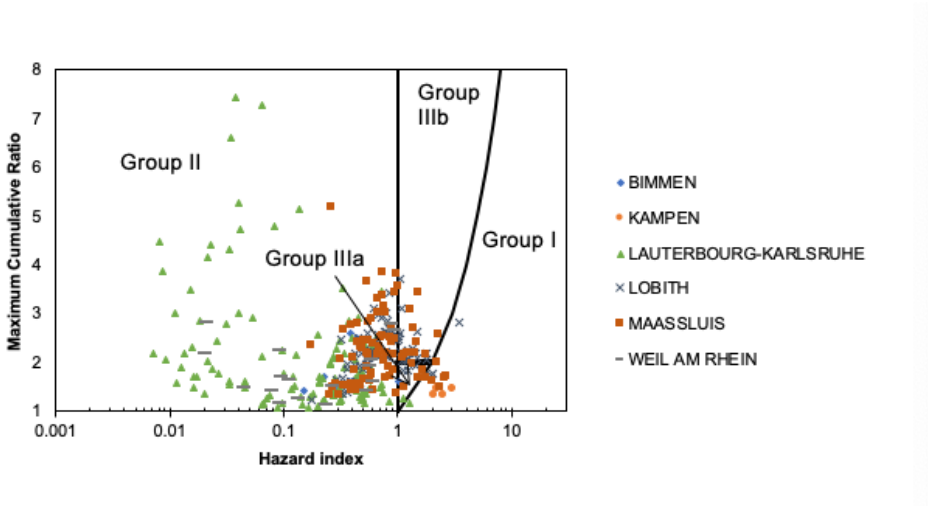


Figure 35: Maximum Cumulative Ratio as a function of the Hazard Index for the Rhine dataset for the more realistic scenario where the TLM-predicted PNEC are used for polycyclic aromatic hydrocarbons instead of the HC5 derived from Posthuma et al. (2019). Different symbols indicate different sampling sites.

Table 19: Overview of statistics for the Hazard index for the mixture exposures for the different groups of substances for the more realistic scenario, after refinement of PAH thresholds.

	All chemicals
Minimum HI	0.01
Maximum HI	3.46
Median HI	0.51
10th percentile	0.07
90th percentile	1.23

Table 20: Overview of the percentage of mixture exposures belonging to the different mixture groups for the more realistic scenario, after refinement of PAH thresholds.

	All chemicals
Group I	3.6%
Group II	83.8%
Group IIIa	2.5%
Group IIIb	10.1%

Table 21: Substances driving the cumulative risks for each of the mixture groups in the Rhine dataset for the more realistic scenario, after refinement of PAH thresholds.

Mixture group	Substance	Maximum HQ	Number of mixture exposures where		
			HQ>1	HQ>0.5	HQ>0.1
Group I (*n=14*) (only substances for which HQ in at least one mixture exposure is >1)	zinc (Zn) dissolved	1.61	11	11	12
	Ethyl-parathion	1.45	1	1	1
	indeno(1,2,3-cd)pyrene	1.22	1	1	1
	diazinon	1.08	1	1	1
Group IIIa (*n=10*) (only substances for which HQ in at least one mixture exposure is >0.5)	zinc (Zn) dissolved	0.92	0	8	9
	Copper (Cu) dissolved	0.91	0	1	9
	chromium (Cr) total	0.78	0	1	9
Group IIIb (*n=40*) (only substances for which HQ in at least one mixture exposure is >0.1)	zinc (Zn) dissolved	0.89	0	18	39
	isoproturon	0.74	0	4	26
	copper (Cu) dissolved	0.59	0	2	40
	chromium (Cr) total	0.44	0	0	33
	diazinon	0.51	0	1	1
	nickel (Ni) dissolved	0.12	0	0	1
	diuron	0.12	0	0	1

3.2.4. PATTERNS OF CO-EXPOSURE IN MIXTURE RISKS

Depending on the sampling site, different combinations of substances are identified to drive the mixture risks in the Rhine dataset. Upstream in Well am Rhein and Lauterbourg-Karlsrue, the pesticide diazinon and copper drive the mixture assessment. However, HQ of both substances are low or concentrations are below the detection limit from 2014 onwards. This indicates that chemical pollution might have improved over the previous decade. Contributions to the cumulative risk in selected samples were observed for the metals zinc, chromium and nickel and the pesticide isoproturon.

In the sampling site Bimmen, it is mainly copper that drives the mixture risks, with in selected samples also substantial contributions of zinc, and chromium. Note that in Bimmen all except one sample are classified under Group II. Downstream the Rhine in the Lobith, Kampen and Maassluis sampling sites more complex mixture occur with copper, zinc, chromium and isoproturon as main drivers and PAH popping up in a limited number of samples.

Overall, this is in agreement with the observation of the PCA performed on the exposure data, where the first PCA-axis was driven by Industrial chemicals/agrochemicals/metals/PAH, although the industrial chemicals seem to influence the cumulative risk less compared to the co-exposure pattern based on concentrations.

Table 22: Overview of substance co-exposure[a] driving the mixtire risk assessment in the different sampling sites of the Rhine dataset (ordered from upstream to downstream sampling site).

	Group I	Group II	Group IIIa	Group IIIb
Weil am Rhein (0/17/0/0)	-	(Copper), (Diazinon), (Nickel)	-	-
Lauterbourg-Karlsruhe (1/110/1/1)	**Diazinon** +Copper	Diazinon, copper, (chromium)	**Zinc** + chromium	**Diazinon,** isoproturon Copper,
Bimmen (0/8/1/0)	-	Copper, (Zinc), (Chromium)	**Zinc** + copper, chromium	
Lobith (3/61/3/18)	**Zinc** + Chromium, copper, isoproturon **indeno(1,2,3-cd)pyrene** + benzo(b)fluoranthene, benzo(a)pyrene, copper, benzo(k)fluoranthene, fluoranthene, zinc	Copper, (Zinc), (Chromium), (Isoproturon)	**Zinc** + copper, chromium, (isoproturon)	**Zinc,** copper, chromium, isoproturon
Kampen (4/67/1/10)	**Zinc** + Chromium, copper, isoproturon **Ethyl-parathion** + copper, methyl parathion, isoproturon	Copper, (zinc), (isoproturon),	**Zinc** + copper, chromium	**Zinc,** copper, chromium, isoproturon
Maassluis (6/67/4/11)	**Zinc** + copper, chromium, (isoproturon), (nickel, benzo(a)pyrene)	Copper, (Zinc), (Chromium), (Isoproturon)	**Zinc** + copper, chromium **Copper** + zinc, cadmium **Chromium** + Copper	**Zinc,** copper, chromium, isoproturon, (nickel, diuron)

[a] Only substances with a HQ>0.1 are considered in the table. Substances printed in bold dominate the cumulative risk, substances between brackets are only significantly contributing in part of the samples considered.

3.2.5. MOA REFINEMENTS: MSPAF$_{MOA}$-APPROACH

The mixture assessment based on the HI/HQ approach that assumes that all substances have the same mode of action, is too simple and might be too conservative. Given the complexity of environmental databases and the possibility of the HI/HQ-aproach overestimating the mixture pressure when several Modes of Action (MoA) are considered, the mixture pressure analysis was further refined by taking into account the different modes of Action (MoA) in the database. This was done by calculating the mixture pressure using a hybrid method between CA and IA,

the $msPAF_{MoA}$-approach. In practice, a Hazard Index was first calculated per mode of action (HI_{MoA}) and then combined into a $msPAF_{MoA}$ to calculate a msPAF for complex mixtures with both shared and different modes of action. In total, 6 different Modes of Action (MoA) were identified among the substances measured in the Rhine dataset, with non-polar narcosis, Ache Inhibition_organophosphate and metallic iono/osmoregulatory impairmente being the most represented. Figure 36 shows the msPAF calculated per MoA. Only a limited number of MoA contribute to the overall $msPAF_{MoA}$ (i.e. for only 3 MoA calculated PAFs were higher than 0.001). On average metallic iono/osmoregulatory impairment shows the highest PAF of all substances.

Refining the mixture pressure assessment with the $msPAF_{MoA}$-approach, resulted in a $msPAF_{MoA}>0.05$ for 4 out of 10 Group IIIa mixtures and for 8 out of 40 Group IIIb mixtures. Under the $msPAF_{MoA}$-approach the mixtures of concern are identified as those mixture exposures for which $msPAF_{MoA}\geq0.05$, because for these mixture exposures it is predicted that at least 5% of the species is affected. The relatively simple $msPAF_{MoA}$ refinement reduces the exposures with high concern for mixture risks in the Rhine dataset to 10% of the total considered mixture exposures.

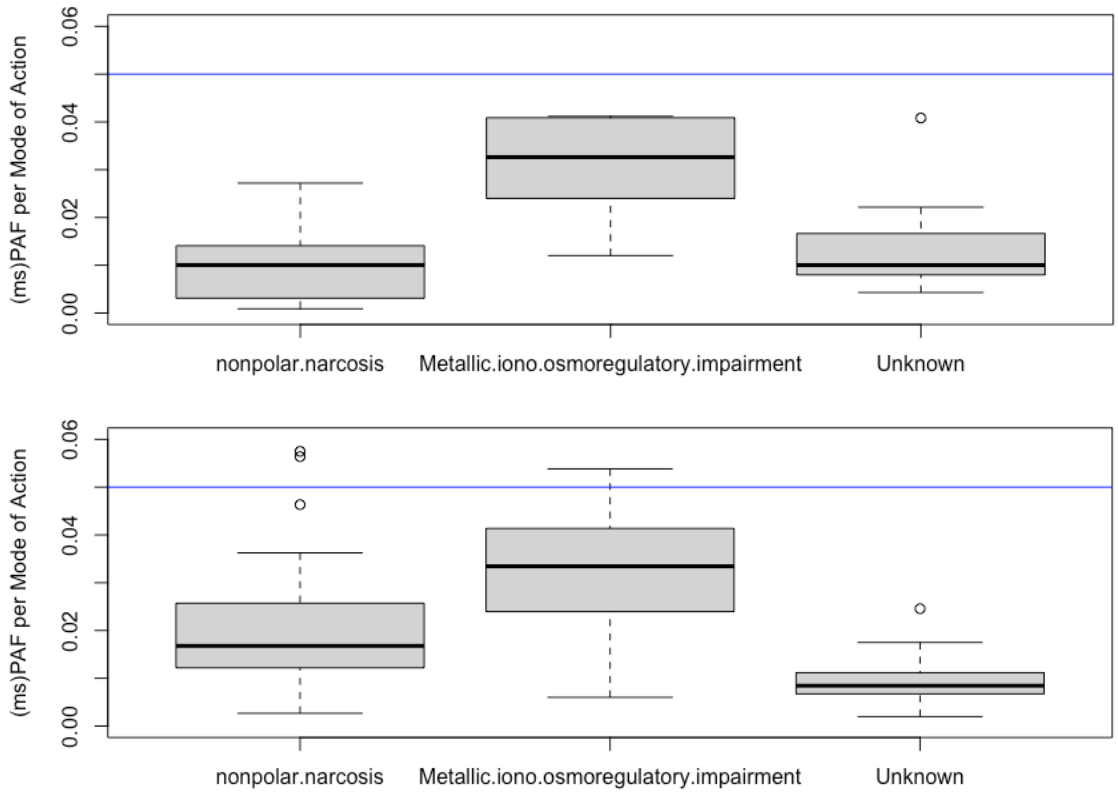


Figure 36: (multi substance) Potentially Affected Fraction ((ms)PAF) for the most important Modes of Action for Group IIIa (upper panel) and Group IIIB mixtures (lower panel).

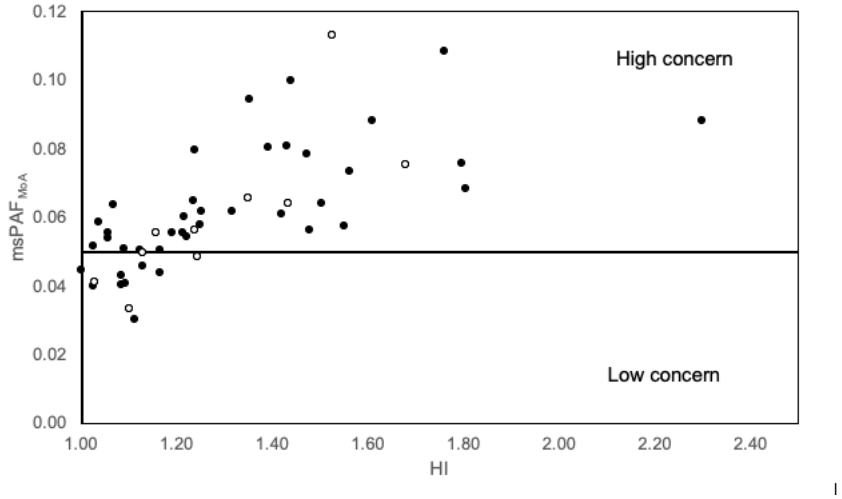


Figure 37: multi substance Potentially Affected Fraction based on refinement per Mode of Action ($msPAF_{MoA}$) as a function of the Hazard Index (HI) for Group IIIa (open symbols) and Group IIIb (filled symbols) mixtures in the Rhine dataset. Under the $msPAF_{MoA}$-approach the mixture exposures with msPAF>0.05 are predicted to be at risk.

3.3.1. ASSESSMENT OF MIXTURE RISKS

Figure 38 shows the cumulative risk of the actual measured substances in the Adour-Garonne dataset for the more realistic scenario (upper panel) and the unrealistic worst-case scenario (lower panel) (realistic with respect to treatment of non-detects, see section 1.2.2). In 1003 samples, none of the 76 measured substance were present above the detection limits. Hence, these samples, were not taken into account for calculation of mixture pressure statistics in the more realistic scenario. The 10th to 90th percentile of the HI, under the more realistic scenario, ranged between <0.01 and 0.88, with a median HI of 0.05 (Table 23). This indicates that the actual risk associated with the measured substances is relatively small for this dataset. However, under the unrealistic worst-case scenario, the cumulative risks in the Adour-Garonne dataset are substantial: the 10th to 90th percentile of HI ranged between 13 and 130, with a median HI of 35 (Table 23).

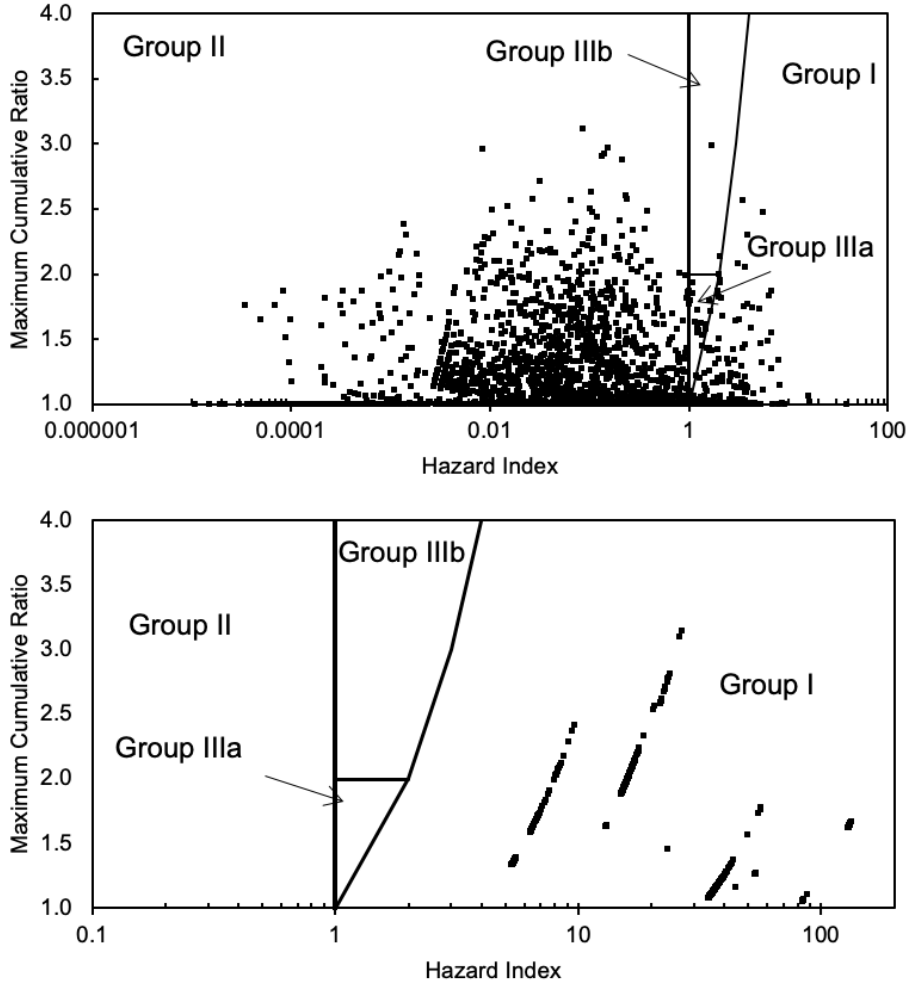


Figure 38: Plot showing the Maximum Cumulative Ratio as a function of the Hazard Index for the Adour-Garonne dataset for the more realistic scenario (upper panel; showing cumulative risks of the detects only) and the unrealistic worst-case scenario (lower panel; showing cumulative risks of the detects at the measured concentration and of the non-detects at the detection limit).

Under the more realistic scenario, the mixture exposures for which there is an actual mixture concern is relatively low, because 91% of the mixture exposures are classified as Group II mixtures. The distribution of the mixture exposures of concern over the different mixture groups is as follows: 8% are classified as Group I, 0.5% to Group IIIa and 0.1% to Group IIIb. Hence, only for a minority of the mixture exposures (<1%), there is a potential concern for co-exposure. For the unrealistic worst-case scenario, all mixture exposures were classified under the mixture Group I, indicating the exceedance of the HC_5 of at least one substance (Figure 38).

Table 23: Overview of statistics for the Hazard index for the mixture exposures for the Adour-Garonne dataset.

	More realistic scenario (<DL=0)	Unrealistic worst-case scenario (<DL=DL)
Number of samples with detected substances	2,554	-
Minimum HI	<0.01	5.3
Maximum HI	38.3	132
Median HI	0.05	35.0
10th percentile	<0.01	13.0
90th percentile	0.9	130

Table 24: Overview of the percentage of mixture exposures belonging to the different mixture groups for the Adour-Garonne dataset.

	More realistic scenario (<DL=0)	Unrealistic worst-case scenario (<DL=DL)
Group I	5.8%	100%
Group II	94%	0%
Group IIIa	0.5%	0%
Group IIIb	0.1%	0%

Over the entire dataset, it is mainly the hormone estrone that is popping up as an important driver of the mixture pressure in almost 70% of the mixture exposures categorized in one of the three mixture groups of concern (i.e. Group I, Group IIIa and Group IIIb). Price et al. (2012) also identified estrone as an important substance in complex mixtures (i.e. Group IIIb mixtures) in a dataset covering a range of European surface waters. In addition, estrone has also been identified as one of the drivers of mixture effects observed in selected bioassays in samples of the Danube used to evaluate effect-based tools (Neale et al. 2015, Königs et al. 2017, Kienle et al. 2019). Estrone is a known estrogenic substance in WWTP-effluents (Kienle et al. 2019). Estrone has also been identified to exceed its PNEC in at least 5% of the environmental samples covering >200 sites in 11 member states (JRC 2016). Cyanide is mainly identified in Group I mixtures, where it exceeds its HC_5 in 21% of the mixture exposures. Cyanide is considered to be a river basin specific pollutant and exceedances of the EQS have been observed in 8 member states (EEA 2018). To a lesser extent also the pharmaceuticals paracetamol and ibuprofen have been identified to exceed HC_5 values, as well as the industrial chemicals chloroacetic acid and bisphenol A. In addition, these substances were also observed to drive the mixture pressure in Group IIIa and/or Group IIIb mixtures. Paracetamol, ibuprofen and bisphenol A have been placed on the list of emerging substances of the NORMAN-network. Bisphenol A is a well-known environmental contaminant that can be found in all surface waters and sediments across Europe in varying concentrations. Bisphenol A has been identified as a priority substance under the WFD directive (JRC 2016). Several studies have listed bisphenol A as one of the drivers of

toxic pressure (e.g. Price et al. 2012, Posthuma et al. 2019, Danube Joint Survey: bisphenol A). For paracetamol, environmental risks were assessed to be relatively low based on monitoring data in 104 individual sites across 4 EU-member states, with 90th percentile Risk Quotient values>0.01). For Ibuprofen, environmental risks were assessed to be higher compared to paracetamol, with 90th percentile RQ of 0.36 across >700 sites in 15 member states. Finally, chloroacetic acid concentrations are associated with relatively high predicted environmental risks across 4 member states, with 90th percentile RQ of 8.3 (JRC 2016).

The driving chemicals under the unrealistic worst-case scenario are visualized in Figure 39. For tributylstannane, estrone, chloroacetic acid and cyanide an exceedance of the HC5 (i.e. HQ>1) was observed in 70-90% of the exposures. For ibuprofen and Irgarol, an HQ>1 was observed in 30% and 4% of the exposures, respectively. For all of these substances, however, measured concentrations were below the detection limit in at least 90% of the mixture exposures, indicating that the cumulative risk is mainly driven by non-detect.

Table 25: Substances driving the cumulative risks for each of the mixture groups in the Adour-Garonne dataset under the more realistic scenario considering only the detect values

Mixture group	Substance	Maximum HQ	Number of mixture exposures where		
			HQ>1	HQ>0.5	HQ>0.1
Group I (*n=207*) (only substances for which HQ in at least five mixture exposures is >1)	Estrone	9.2	135	143	145
	Cyanide	14.8	43	50	67
	Paracetamol	3.0	11	13	23
	Chloroacetic acid	15.9	10	14	17
	Ibuprofen	3.2	9	11	18
	Bisphenol A	3.0	8	9	11
Group IIIa (*n=19*) (only substances for which HQ in at least two mixture exposure is >0.5)	Estrone	0.97	0	11	12
	Bisphenol A	0.99	0	3	3
	Ibuprofen	0.91	0	3	5
	Paracetamol	0.79	0	2	6
	Chloroacetic acid	0.67	0	3	7
Group IIIb (*n=2*) (only substances for which HQ in at least one mixture exposure is >0.1)	Cyanide	0.96	0	2	2
	Estrone	0.70	0	2	2
	Paracetamol	0.14	0	0	2

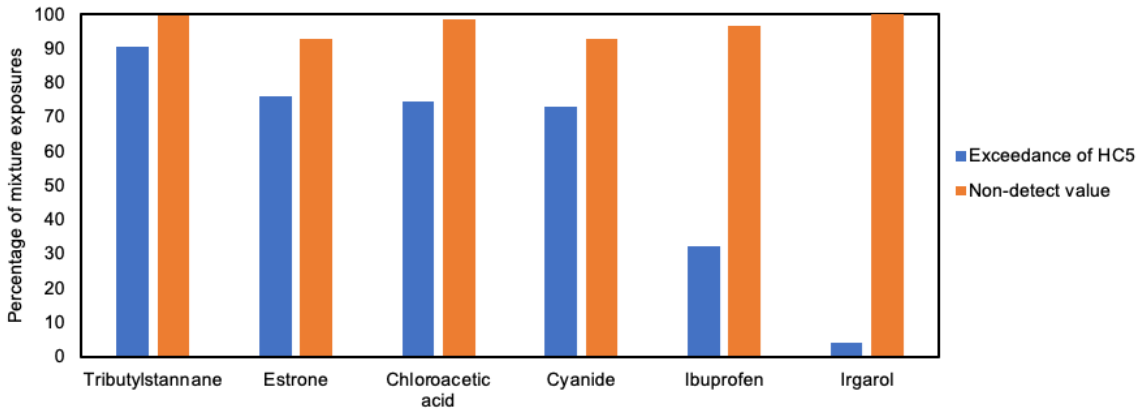


Figure 39: Percentage of mixture exposures with an exceedance of HC5 (blue bar) and non-detect values (substances is present below its detection limit), for the substances driving the unrealistic worst-case scenario in the Adour-Garonne dataset.

3.3.2. PATTERNS OF CO-EXPOSURE IN MIXTURE RISKS

In the Adour-Garonne dataset, different combinations of driving chemicals can be identified in the Group III mixture, mostly related to catchment difference. In the Charente catchment, the Group IIIa mixtures are driven by the risks associated with both estrone and chloroacetic acid. In the Garonne and Adour catchments, estrone is the common risk driver in Group IIIb mixtures, in combination with chloroacetic acid and/or cyanide. In the Lot catchment, the Group IIIa mixtures are mainly driven by the pharmaceutical ibuprofen in combination with paracetamol, while in the Dordogne bispenol A drives the mixtures risks in combination with paracetamol. The more complex mixtures in the two Group IIIb samples are driven by a combination of estrone, paracetamol and cyanide.

3.3.3. MOA REFINEMENTS: MSPAF$_{MOA}$-APPROACH

In total, 22 different Modes of Action (MoA) were identified among the substances in Group IIIa and Group IIIB mixtures in the Adour-Garonne dataset, with non-polar narcosis, polar narcosis and diester toxicity being the most represented. Only a limited number of MoA contribute to the overall msPAF$_{MoA}$ (i.e. for only 8 MoA calculated PAFs were higher than 0.001). On average estrogenic shows the highest PAF of all substances.
Refining the mixture pressure assessment with the msPAF$_{MoA}$-approach, resulted in a msPAF$_{MoA}$>0.05 for 3 out of 19 Group IIIa mixtures. Under the msPAF$_{MoA}$-approach the mixtures of concern are identified as those mixture exposures for which msPAF$_{MoA}$≥0.05, because for these mixture exposures it is predicted that at least 5% of the species is affected.

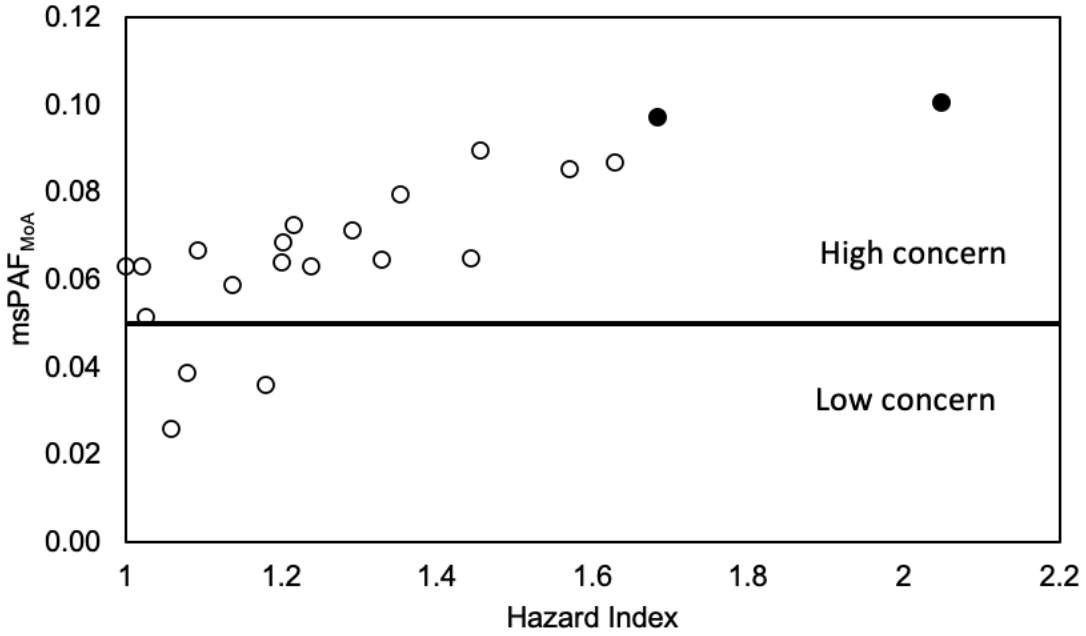


Figure 40: Multi-substance Potentially Affected Fraction based on refinement per Mode of Action ($msPAF_{MoA}$) as a function of the Hazard Index (HI) for Group IIIa (open symbols) and Group IIIb (filled symbols) mixtures. Under the $msPAF_{MoA}$-approach the mixture exposures with msPAF>0.05 are predicted to be at risk.

4. TECHNICAL ANNEX D: BIOLOGICAL IMPACT OF MIXTURES

4.1. COUPLING CHEMICAL AND BIOLOGICAL MONITORING DATABASES

4.1.1. METHODOLOGY

Biological status of surface waters is monitored under the auspices of the **Water Framework Directive**. The biological status is one part of the water quality, in addition to chemical water status. Member States are required to monitor and report the biological status of their surface waters. Typically, this is done using **biological indices** for different groups: most often for macroinvertebrates and fytobenthos as these are easy to monitor, but also fish, macrophytes and phytoplankton. These biological indices are of value to assess the real biological impact/ecological status of chemicals and mixtures. Biological indices are easier to interpret than the abundances of individual species, which are typically not reported anyway: interpretation of what species are sensitive or indicators of good water quality requires expert knowledge while the biological indices translate this to an interpretable value. In the environment, biological status is determined by a multitude of factors e.g. nutrients, hydromorphological properties and physico-chemistry of the water. This project will evaluate the relative contribution of chemical pressure on the biology.

Coupling the available chemical monitoring databases with biological databases requires to find **corresponding samples** in both databases i.e. samples taken at the same location in a comparable time frame. The impact of chemical exposure often only manifests some time after it occurs (chronic effects). From an environmental perspective, these long-term effects are also the most critical. Therefore, the impact of chemicals was assessed on the biological status of the following year(s). In practice, this means that samples in the biological database were coupled with chemical samples from the year(s) before.

4.1.2. DATA PROCESSING

Three databases were explored for assessing the biological impact of mixtures: Waterbase, Adour-Garonne and Flanders. Depending on the database, multivariate (RDA) or univariate (GLM) statistical techniques were used.
In the databases, not all biological indices are available for each row. As was the case for PCA, a complete dataset is required for RDA i.e. for each row (sample), all columns (biological indices) should have a value. Again, data processing is thus required to resolve this. If a reasonable dataset with multiple biological indices was not possible, the data were aggregated by taking the minimum value of available indices. This resulted in one value per sample, which was considered the worst case biological status for that sample. Additionally, biological indices do not necessarily have the same scale e.g. the number of sensitive EPT taxa cannot directly be compared to a derived index such as the Shannon-Wiener diversity index. Standardisation of all indices allowed each biological index to equally contribute to the analysis (in the case of RDA) or ensured comparability between the indices (when taking the minimum of available indices). The data processing per database is discussed below.

The Waterbase includes 14 biological indices for 28,941 rows, but the indices for macroinvertebrates and phytobenthos are clearly the most available (Table 26). Despite this large number of indices, there are typically only one or a few indices available per sampling location and period. This resulted in a dataset with many missing values, making a RDA impossible. Therefore, the minimum index per sampling location and date was selected and a GLM analysis was performed.

Table 26: Biological indices in the Waterbase.

Index	Count
PhytobenthosEQR_H	5
PhytoplankotnEQR_A	54
MacrophyteEQR_E	56
PhytoplanktonEQR_G	57
PhytobenthosEQR_A	68
MacrophytesEQR_G	75
FishEQR	84
PhytoplanktonEQR_E	84
InvertebrateEQR_H	173
InvertebrateEQR_A	446
InvertebrateEQR_E	1,270
PhytobenthosEQR_G	5,961
PhytobenthosEQR_E	7,537
InvertebrateEQR_G	13,071

To ensure the largest possible dataset, chemical measurements performed within 1.11 km (2 digits of the coordinates) of the biological sampling location and from the same water body were considered. No exact dates are reported for the biological samples, but a time period of a few months.

Coupling of the biological and chemical database proved difficult. There is minimal overlap in the sampling frequency: the biological data was available for the period 2006-2015 while the chemical data is nearly absent for this period (Figure 41). No corresponding chemical data was available for the year before the biological samples. Allowing the chemical measurements to be up to 5 years old, still only resulted in a dataset of 254 rows. Therefore, further analysis on the Waterbase dataset was not possible.

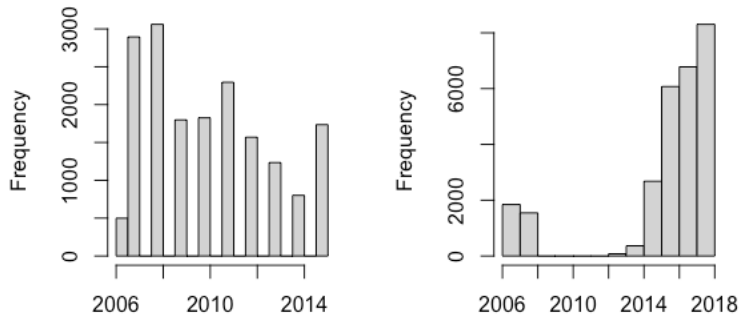


Figure 41: Year availability in the biological (left) and chemical (right) datasets of the Waterbase.

4.1.2.2. ADOUR-GARONNE

The Adour-Garonne databases contain, in addition to chemical concentrations, measurements of the physico-chemistry of the water (e.g. pH, N, P, conductivity) and biological indices. Chemical and physico-chemistry data of the year before were coupled with the biological indices. The toxic units of the chemical concentrations (using the chronic HC5) were summed to derive a conservative (worst-case) estimate of the mixture pressure. After data processing, there was a high correlation (0.99) with the maximum toxic units, indicating that the mixture pressure was typically dominated by one chemical.

31 indices are available in the Adour-Garonne database, of which 26 are regularly reported. Data exploration revealed that typically only one index was reported per sample. For RDA, a complete multivariate dataset could not be constructed. A GLM analysis was thus performed. The data were aggregated to take the minimum biological index for that sampling location, from here on referred to as the biological status index. Before aggregation, the indices were standardized by subtracting the mean value of the index for all samples and dividing by the standard deviation.

Further data exploration revealed that the biological status indices were normally distributed. Therefore, the gaussian distribution was chosen for the GLM. Many of the physico-chemistry variables have many low values and a small number of higher values and were therefore log-transformed to allow a better spread of the data. The following variables were log-transformed: chemical pressure (log(C + 0.01) to avoid issues with zeros), Kjeldahl N, Conductivity, BOD5, Suspended matter and total P. Following variables were not transformed: saturated oxygen, pH, temperature and dissolved oxygen. Temperature led to issues with multicollinearity, which should be avoided for GLM analyses. Therefore, temperature was left out of the analysis. Dissolved oxygen was highly correlated with saturated oxygen and was also left out of the GLM analysis.

4.1.2.3. FLANDERS

Eleven biodiveristy indices were available in the database, covering three ecological groups: phytobenthos (2 indices), macrophytes (5 indices) and macroinvertebrates (4 indices). An overview of the indices is given in Table 27.

Table 27: Biodiversity indices in the Flanders (VMM) database.

Ecological group	Biodiversity index	abbreviation
Phytobenthos	Impact associated species	IA
	Impact sensitive species	**IS**
Macrophytes	Score for growth form	GV
	Score for the development stage of the vegetation	VO
	Disturbance score	V
	Score for specific types	TS
Macroinvertebrates	Shannon-Wienser diveristy index	Shannon.Wiender.Diversity
	Number of sensitive taxa	Number.of.Sensitive.Taxa
	Number of taxa	Taxa.Richness
	Score calculated to indicate tolerance of taxa	Tolerance.score
	Number of sensitive EPT taxa	EPT.Richness

Table 28: Pollutants measured in the Flanders dataset.

Metals	Pesticides
Ag	Diuron
As	Linuron
B	Metalochlor
Ba	Atrazine
Be	Desethylatrazine
Cu	Simazine
Cd	Terbutylazine
C	Chloridazon
Cr	Carbendazim
Hg	Pirimicarb
M	Flufenacet
Ni	aminomethylphosphonic acid
Pb	Glyfosaat
Sn	Mecoprop
Se	2-methyl-4-chlorophenoxyacetic acid
Sn	Dichloorprop
Te	4-(2,4-dichlorophenoxy)butyric acid r
Ti	Fluroxypyr
	Diflufenican
	Ethofumesate
	Metribuzin
	Oxadiazon

Data was available from 2007 to 2015. The Flanders databases for physico-chemistry, pollutants and biodiversity indices have been coupled before in the context of the ELMO project[4]. The same methodology was followed in this project. For RDA analysis, a complete dataset is required. Because the metal concentrations and organic pollutants are monitored in separate monitoring campaigns, both were evaluated individually. For the organic pollutants, only the pesticides were selected. After coupling, the dataset consisted of 1,510 rows when using the metal concentrations and 572 rows with the pesticide concentrations.

To account for mixture toxicity, the toxic units of the metals were summed. As effect concentrations, the ecological reference values were taken from Meclas (www.meclas.eu). To

[4] Bennetsen et al. 2014; https://www.life-belini.be/en/portfolio-item/integral-analysis-of-water-bodies-using-an-aquatic-ecological-model/

account for background concentrations, the added concentration approach was used. For pesticides, because all pesticides were only measured in a small subset of the samples (0.025%), no toxic units were calculated but the pesticide concentrations were summed to get an indication of their potential role. The impact of pesticides could thus not be analysed thoroughly for this dataset.

4.2. BIOLOGICAL IMPACT

4.2.1. ADOUR-GARONNE

After model selection, the final model included organic nitrogen (Kjeldahl), conductivity, chemical pressure and total phosphorus as significant variables (Table 29). All were found to have a negative impact on the biological status index, meaning that higher values of these variables decrease the biological status. The role of chemical pressure is however subservient to the influence of organic nitrogen (four times higher impact), conductivity (two times higher impact) and similar to the impact of phosphorus. Interestingly, biological oxygen demand and suspended matter were not found to impact the biological status.
Model assumptions for the final model were validated based on visual inspection of the residuals, QQ-plots and observed versus predicted plots (Figure 42 and Figure 43).

Table 29: Variables retained in the final GLM model, their estimated coefficient and associated p-value.

Variable	Estimated coefficient	p-value
(Intercept)	-0.242	0.181
SumTU_chronicHC5	-0.118	**1.03E-09**
N.Kjeldahl	-0.458	**4.11E-07**
Conductivity	-0.205	**4.13E-07**
P.t	-0.124	**0.002**
SaturatedO	-0.004	**0.001**

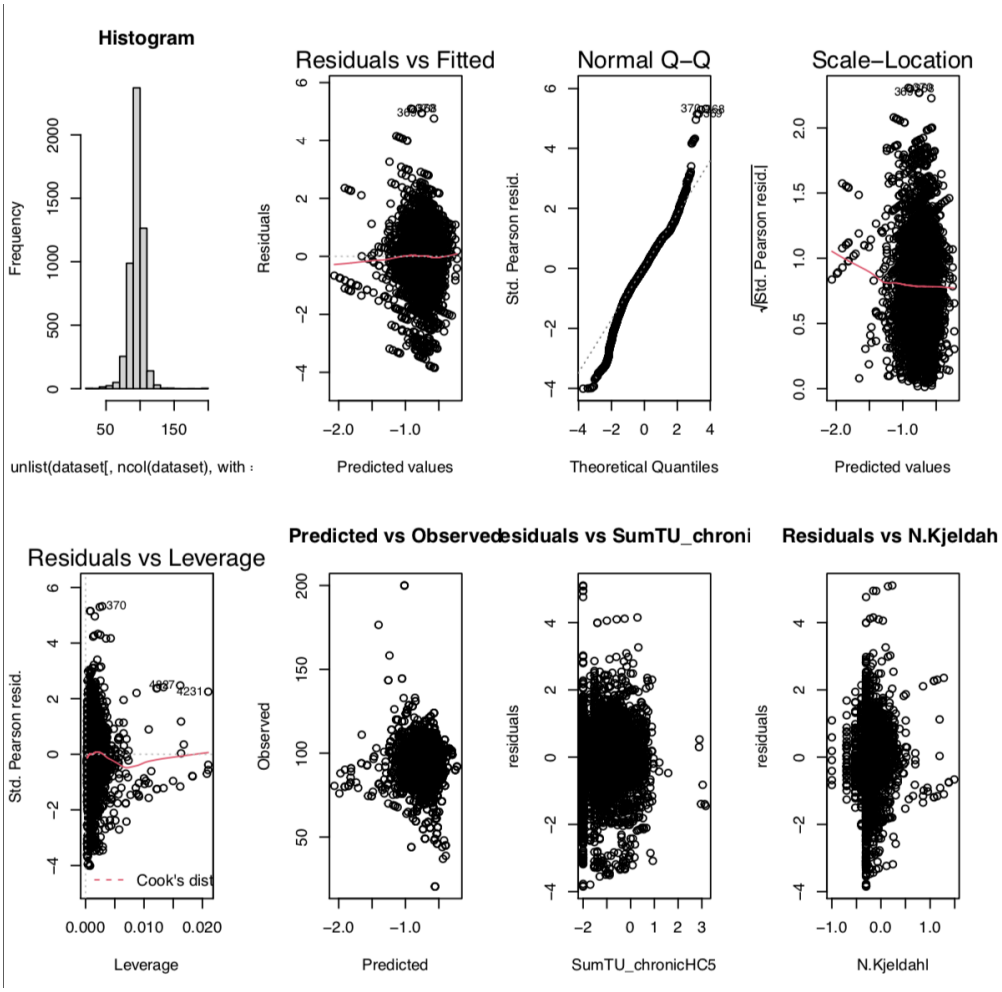


Figure 42: Model assumptions for the GLM analysis on the Adour-Garonne dataset.

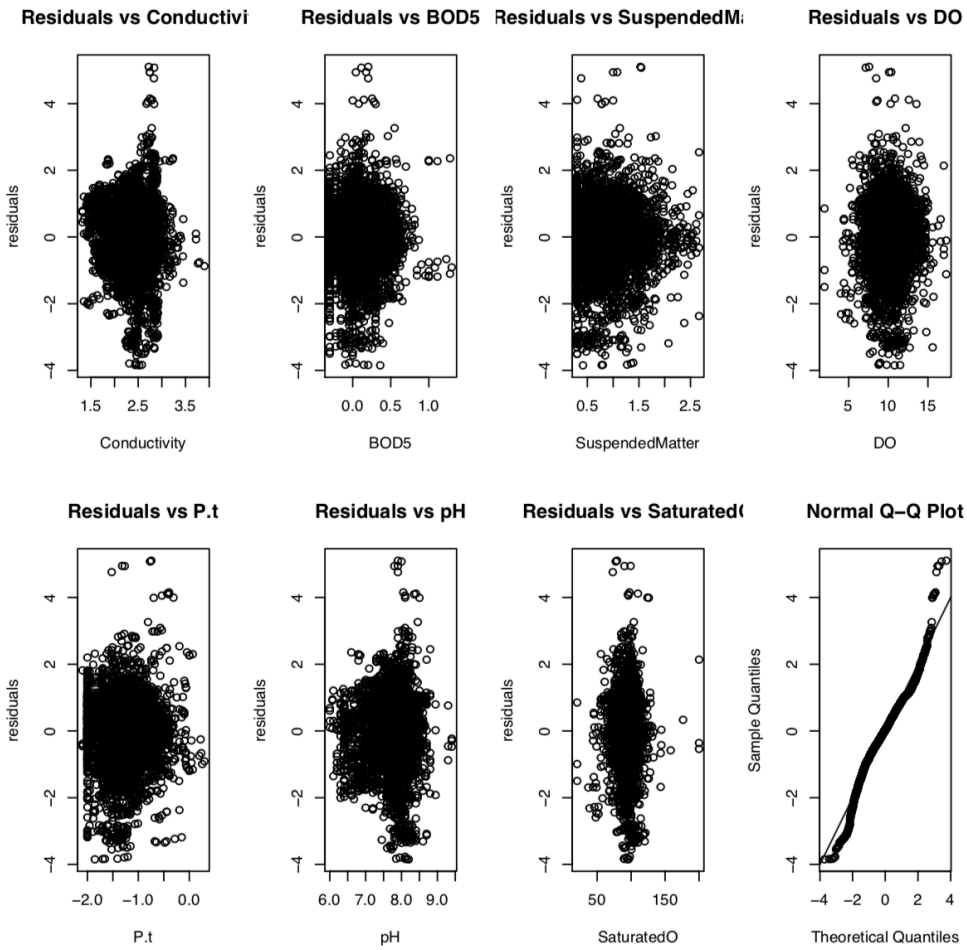


Figure 43: Model assumptions for the GLM analysis on the Adour-Garonne dataset.

4.2.2. FLANDERS

The variance explained by the RDA was rather limited. For the analysis with metals, 32% of the variance was explained, for pesticides 43%. Considering metals, the physico-chemistry (especially nutrients nitrogen and phosphorus) was the main contributor to the biodiversity. Metals only contributed a minimal fraction to the biodiversity (1%) compared to physico-chemistry (31%). In line with this, the RDA scores for metals were low and of low importance compared to nitrogen and phosphorus.

The sum of all measured pesticides was found to be a more relevant contributor to the biodiversity, explaining 2% of the variance independently and 20% of the variance in combination with physico-chemistry. The summed pesticides were the main determinant of the first component, indicating its importance for measured biodiversity indices. In this analysis, the summed concentrations of the pesticides were used and this does thus not account for potential risks of pesticides (using toxic units). In combination with the low number of samples (572), this result suggests that this warrant further investigation but cannot be used to interpret the potential impact of pesticides.

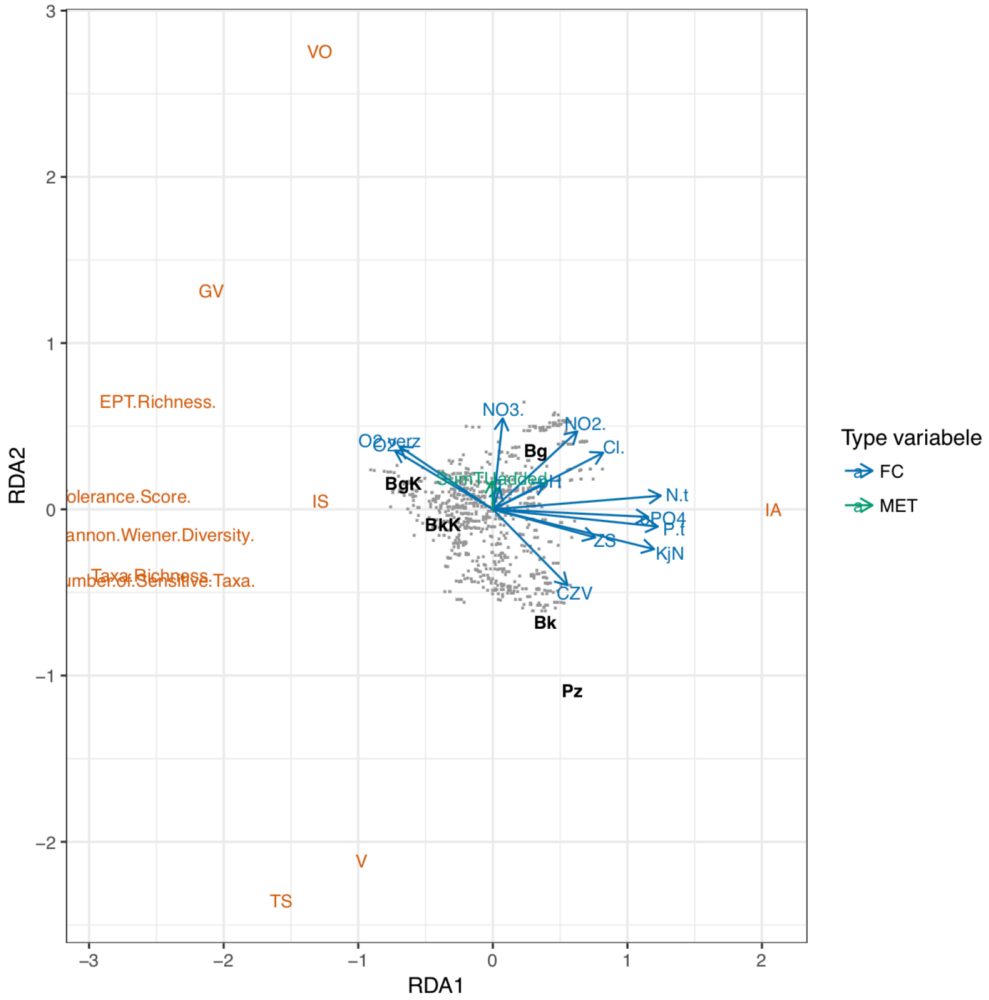


Figure 44: Triplot for the RDA on the Flanders dataset with metals. FC = physico-chemistry, MET = metals.

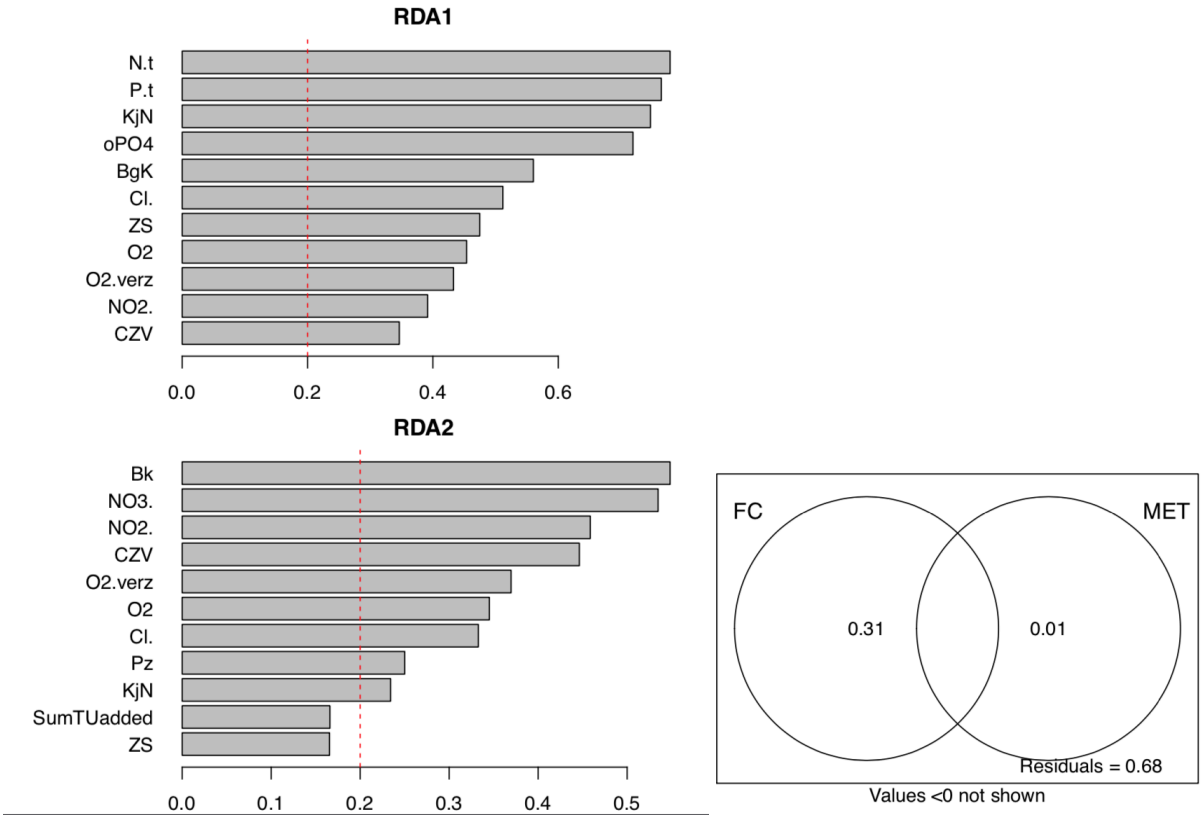


Figure 45: The loading (indicates importance) of each variable (left) and the variance explained (right) for the RDA on the Flanders dataset with metals. FC = physico-chemistry, MET = metals.

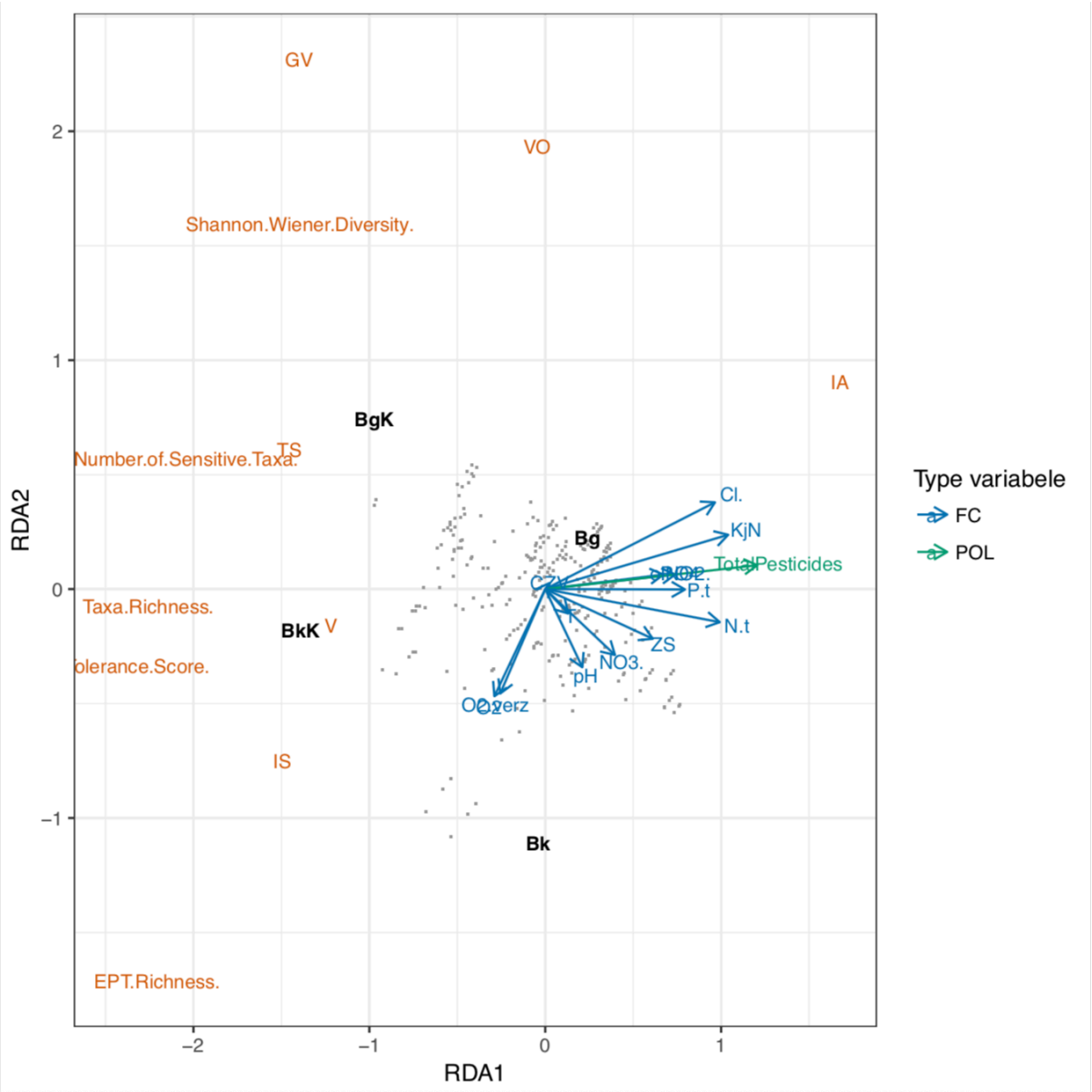


Figure 46: Triplot for the RDA on the Flanders dataset with pesticides. FC = physico-chemistry, POL = pollutants i.e. pesticides.

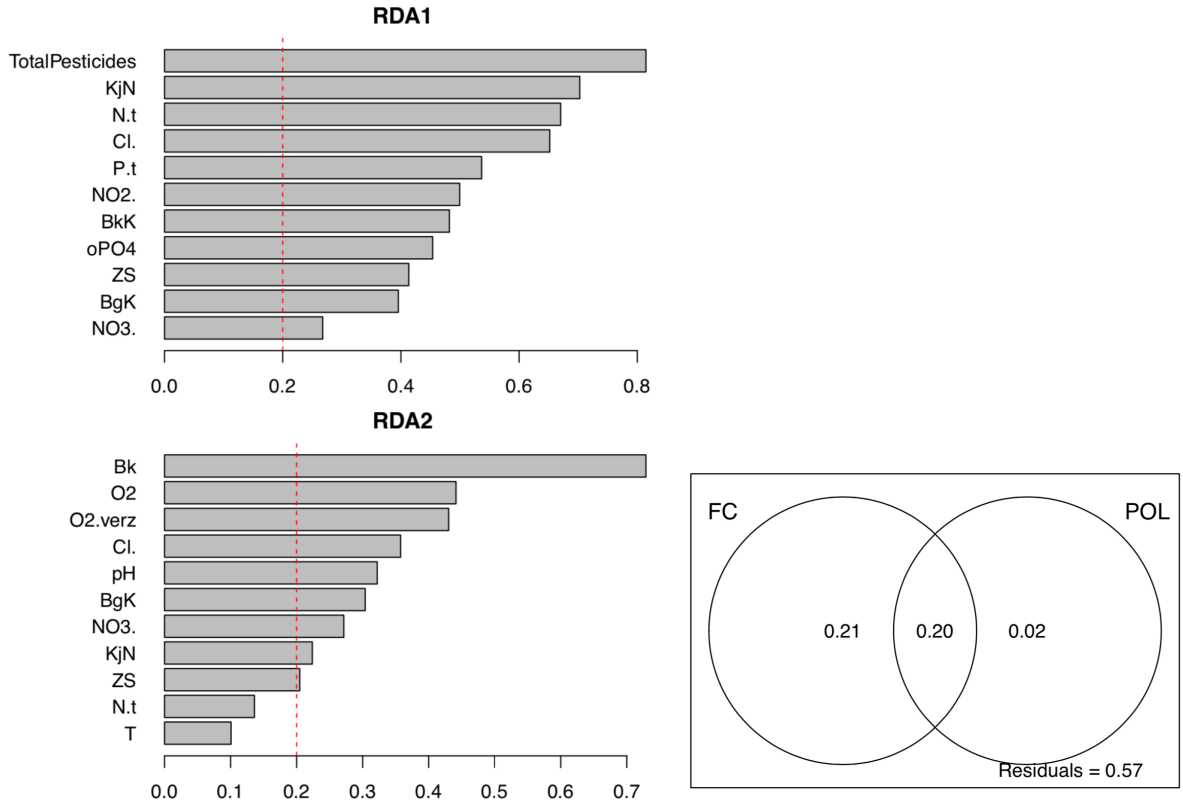


Figure 47: The loading (indicates importance) of each variable (left) and the variance explained (right) for the RDA on the Flanders dataset with metals. FC = physico-chemistry, POL = pollutants i.e. pesticides.

5. TECHNICAL ANNEX E: IDENTIFICATION AND UNDERSTANDING CO-EXPOSURE FOR HUMAN HEALTH

See separate document.